COMPARISON BETWEEN MANUAL AND AUTOMATED METHODS OF DECALCIFICATION OF TEETH-AN IN VITRO STUDY

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In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY & MICROBIOLOGY

2016 – 2019

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This is to certify that the dissertation titled ''**COMPARISON BETWEEN MANUAL AND AUTOMATED METHODS OF DECALCIFICATION OF TEETH-AN IN VITRO STUDY''** is a bonafide work done by **Dr. K. GAYATHRI,** Postgraduate student, during the course of the study for the degree of **MASTER OF DENTAL SURGERY** in the specialty of **DEPARTMENT OF ORAL PATHOLOGY AND MICROBIOLOGY,** Vivekanandha Dental College for Women, Tiruchengode, during the period of 2016-2019.

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PLACE OF STUDY	ELAYAMPALAYAM, TIRUCHENGODE, NAMAKKAL DISTRICT.
DURATION OF THE COURSE	3 Years (2016-2019).
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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3424938/
http://aimdrjournal.com/pdf/Vol2Issue3_17_OA_Shahid_2_3_25.pdf
http://www.ijdr.in/article.asp?issn=0970-9290;year=2013;volume=24;issue=3;spage=302;epage=308;aulast=Prasad
https://www.slideshare.net/DRBHUVANNAGPAL/24-decalcification-lambert
https://nios.ac.in/media/documents/dmlt/HC/Lesson-06.pdf
http://www.jlponline.org/article.asp?issn=0974-2727;year=2016;volume=8;issue=2;spage=106;epage=111;aulast=Srinivasyaiah
http://www.ijmdent.com/article.asp?issn=2229-6360;year=2017;volume=7;issue=2;spage=94;epage=100;aulast=Kadashetti
http://docplayer.net/37230604-Decalcification-module-6-1-introduction-objectives-6-2-decalcification-6-3-different-methods-of-decalcification-notes.html

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INTRODUCTION

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Decalcification is a process of complete

removal of calcium salt from mineralized tissues

like bone, teeth and other calcified tissues.

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In routine histopathology,

decalcification of teeth is an essential and important step during tissue processing,

and plays an important role in histological diagnostic techniques. Various acids or chelating agents are used for decalcification in the laboratories for microscopic examination of hard tissues.

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The goal of decalcification is to remove calcium salts from the mineralized tissues,

while preserving the organic portions and

preparing

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them for further sectioning of the histological specimen.

Any acid, even if properly buffered, affects tissue stability. These effects depend on the solution's acidity and duration of the decalcification process.

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The

factors influencing the speed of

decalcification include decalcifying solution concentration, temperature, stirring and tissue suspension. Decalcification is performed by chemical solutions, which employ acids (acids may be

divided into strong and weak acids or chelates). It is thought to

be emphasized that fixative agents that contain acid in their composition, such as formalin (that contains formic acid), may also

be

able to act as decalcifying agents if the acid component is not neutralized.

Quicker decalcification is

essential for faster diagnosis of hard tissue pathology.

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So, an ideal decalcifying agent should be fast, be good and do good.

Apart from properties of decalcifying agents, the method of decalcification also affects the outcome of the decalcified specimen.

Automated decalcifying instruments are available, which enhances the process by constant rotation and stirring of the specimen and maintaining the temperature. Thus, the aim of our study is to compare automated and manual tissue processing methods, to know the best best method for studying the decalcified specimen.

AIM & OBJECTIVES • The aim of the present study is to compare between manual and automated methods of decalcification.

• The objective of the study is to find any difference between manual and automated methods of Decalcification.

REVIEW OF LITERATURE

According to Drury and Wallington (1980), strong

acids, weak acids , chelating agents have been used to remove calcium ions from hard tissues . Although decalcification by strong acids is rapid, it damages tissue and affects tissue stainability. Decalcification with weak acids, such as formic acid

can

preserves tissue details, but is time consuming. Chelating agents, such as EDTA, do not damage tissues , but they require a long time (Callis 2008).

According to Verdenius and Alma (1958),

Decalcification can be done by applying heat, agitation, vacuum and electric current . Microwave energy (Sangeetha et al 2013) and ultrasonic cleaners (Hatta et al. 2014) also have been used to accelerate decalcification.6

ultrasonic cleaners (Hatta et al. 2014)

Microwave energy (Sangeetha et al 2013) and also have been used to accelerate decalcification.6 The speed and quality of histological processes including the decalcification of teeth and bone,can be better enhanced if they are carried out in a microwave oven.(Boon & kok, 1988).

Balaton and Loget (1989) have also

reported that the decalcification of bone is accelerated about ten times compared with that ambient temperature.7

Verdenius and Alma in 1958 compared other methods of decalcification and their modifications namely, different temperatures ,different acids,use of electric current,Vaccum,.and physical movement.

The following methods have been described in the literature.

Heat: Heat is a method to hasten the decalcification process.4

A chemical

process is accelerated two to three times for every 1000 C.

of temperature, and Murayama, Suzuki, and Itoh (1937) revealed that with increased temperature the decalcification process takes place within an even

smaller duration of

time.8 Heat increased the diffusion of reaction products,which decreased the decalcification time(verdenius and Alma 1958).

In their study, Verdenius and Alma(1958) showed that

Heat is also known to accelerate decalcification as it raises the diffusion rate and increases the chemical reaction rate. They also observed that the time used for decalcification decreased as temperature was increased from 13oC to 25oC to 40oC . Similarly, intermittent heating to 45oC in a hot air oven showed fastest decalcification of teeth specimens.4 Heat reduces the liquid viscosity ,thereby speeding the rate of diffusion of reagents in to and out of the tissue .9Agitation was slower than heating by 1 day, but it can preserve the Pulp cellular details .4 In this method

the possibility of interaction between the object and the surrounding decalcifying fluid is increased.8

Diffusion is a key factor

in histoprocessing,.

Boon et al., (1986) describe how microwaves increases diffusion of reagents into the tissue, and out of it. As explained in some detail by Kok (1986), the rise in temperature increases diffusion rates. The key formula for diffusion is = 2Dt. Here x = the net distance covered by a particle in solution in a particular direction, t = time period during which the diffusion process takes place, D = the diffusion constant, characteristic of solute and solvent. The brackets signify 'average', hence in words the formula expresses: the average squared distance covered by a particle in solution is proportional to the diffusion time. This indicate the need to maintain biopsies thickness small: three times as thick means nine times as long for comparable effects. Note that width and length of the biopsy hardly matter. To obtain the same findings in a 10 times shorter time, one must increase D by a factor of 10. Now D is highly temperature dependent. Furthermore, it is advantageous to work with small molecules.10

Vacuum: The process of decalcification can be characterized as follows

by creating a vacuum:

Insoluble calciurn-salts+acid o-soluble calcium salts+CO2. The carbon dioxide, rapidly extracted,

would cause disturbance in

the balance of the chemical equilibrium, and, according to Le Chatelier,

it can

result in an acceleration of the reaction. Waerhaug (1949), however, showed that there will be a more rapid contact between the specimen and the surrounding fluid, the CO2 bubbles can being rapidly removed. Waerhaug (1949), Frank and Deluzarche (1950),and Engelbreth-Holm and Plum (1951) also

showed a considerable

shortening of the time of decalcification. According to Molenaar (1957) only, the decalcification process only slightly accelerated.8

In a study by waerhaug,bone and teeth were decalcified rapidly

under vaccum

and

the time taken for decalcification was reduced to one –tenth.5

Electric Current: under

the influence of an electric field ,the Ca ions liberated by the decalcifying fluid will be removed more rapidly. Richman, Gelfand, and Hill (1947), Ducey and Shippy (1950), Dolan (1951), and Scheliga (1952) revealed that the proces of decalcification is greatly accelerated, whereas the stainability will remain unchanged.

According to various group of investigators (Lillie, Laskey, Greco, Burtner, and Jones, (1951),

the increased

speed of reaction was arised to result only from the rise in temperature due to the

passage of the electric current. These results were confirmed by Molenaar

in 1958.8 In 1937 Murayame

Suzuki and, Itoh revealed that with increased temperature for the process of decalcification actually takes place within a shorter time.

Goncalves &Oliverio used an electric decalcification techniques, with alternate chain, increasing the decalcification velocity.According to these authors,this process promotes molecule shaking,resulting

in an increase of the decalcification process.3 The

study conducted by Nylen et al. (1963) showed that increased enamel calcification was due to an increase in crystal size. However, there is disagreement on the morphology and decalcification pattern of rat enamel crystals (Warshawsky, 1987a,b; Warshawsky et al., 1987).11 The use of Microwavess as a method to fix tissue for neurochemical analysis is well established (Stavinoha et al., 1970; Schneider et al., 1982) .12 In 1995, Boon et al reported that it was possible to produce significant acceleration of tissue processing by introducing microwaves . In 1998, Visinoni et al, followed up with a description of the first microwave tissue processor that completed processing in between 30 and 120 minutes, depending on the thickness of the specimen .13 In 2003,

Correa et al.

reported that the decalcification process causes important morphological alterations in the tissues such as vacuolation edema, and ruptures not contributable to

the pathologic condition.14

Zappa J et al., (2005) compared the decalcification of 288 carious and non-carious teeth with 10% EDTA, 10% EDTA-TRIS,

and electrolytic decalcification with Romeis fluid.

They have concluded that Romeis fluid was the best for routine histopathology diagnosis and EDTA was the best for educational and research purposes where time is not a constraint. In 2011 Nadaf A,

Madhura S, Radhika M B,Parimala ,Sudhakara M. suggest that Decalcify

thirty teeth in 5%nitric acid, 10% nitric acid, 10% nitric acid-10% formalin and10% nitric acid – 20% formalin at 55°C.

Decalcified teeth of Group containing 10 % HNO3 and 20% formalin proved to be faster among the three groups while maintaining perfect tissue details.15

Sanjai K,

Kumarswamy J, Patil A, Papaiah L, Jayaram S, Krishnan L(2012)

have compared

the rate of decalcification of six decalcifying agents and their effect on staining

of dental tissues. They also studied 5% nitric acid, Pereneyi's fluid, formal nitric acid, 5% trichloroacetic acid, EDTA, and 10% formic acid They reported that EDTA was the slowest but preserved soft tissue integrity and staining characteristics very well, while 5% nitric acid was fastest but least considerate to the tooth structure5. Prasad P and Donoghue M (2013) have conducted a study on six DAs: 10% formal nitric acid,8% formal nitric acid, Pereneyi's fluid and EDTA, on posterior mandible of rat. They found that both 8% and 10% formal nitric acid gave quick decalcification and good tissue results1.

Apart from the properties of decalcifying agents, the method of decalcification can also affects the outcome of the decalcified specimen Decalcification is the commonly employed technique in histopathology laboratories as a part of calcified tissue preparation for the microscopic examination.2 Chemical agents are commonly used for routine decalcification procedures, though some agents adversely damage the tissue integrity and staining properties.16

Tooth decalcification is a time consuming process. It takes days to weeks and tissue preservation structure depends on the quality of the demineralization process2.

The degree of decalcification was determined by the lack of resistance to cutting the specimen with a razor blade. The time required for decalcification differs directly with the size of specimen.17 when enzymes histochemical localization is to be done on decalcified hard tissues,it is important to perform the decalcification in the shortest period time possible in order to preserve as much of the enzyme as possible in their original state 18. Histologic studies of mature hard tissues, and teeth,

bone

have been conducted for the most part with formalin fixation and acid demineralization.19-23 The diffusion plays an important role and that the decalcification progress is significantly

affected by the rate of diffusion of decalcifying solution in the specimen.24 Research has been done in

the introduction of new decalcifying as well as modification of known decalcifying agents to meet good decalcifying agent criteria ,which should ensure calcium removal completely at a

good

speed with minimal damage to cells and tissues.2

Solid tissues need to be processed,to preserve their structures,and eventually impregnated with an appropriate hardening substance to permit making thin slices suitable for microscopic evaluation.Inorganic component of teeth can be studied in ground sections, but decalcification is required to study the organic components . Moreover, the pulpal soft tissue which otherwise is difficult to appreciate in the ground sections, can be easily assessed in decalcified sections . Decalcification is routinely used technique in most histopathological laboratories for the microscopic examination of calcified tissues. Decalcification may be regarded as any treatment which destroys the inorganic phase,with the removal of the essential element calcium,in such a way so as to leave the organic portion sufficiently intact to be handled and sectioned by normal methods.25 Correlation of cytological and morphologicall features with immunophenotyping is difficult.26 A number of histological methods have been adapted over the

many

years to analyze normal and pathological features of calcified tissues.27 For almost 100 years,the steps followed to prepare tissues for microscopic review have remained practically unchanged.Decalcification was checked by serial roentgenograms at various stages of the process.28

The choice of decalcifier is affected by four interdependent factors;

urgency of the case,degree of mineralisation,extent of the investigation and staining techniques.

Gray (1954) listed over 50 different mixtures.

Many of

these mixtures were developed for special purposes with one used as a fixing and dehydrating reagents .e.g,Buffer salts ,chromic acid ,formalin or Ethanol, intended to counteract the swelling effects

of

acids on tissues.The process of dehydrating,clearing and impregnating agent required to obtain paraffin sections is time consuming. All three steps are based on diffusion,and the aim is to replace one reagent by another.29 Lineweaver-burk plots revealed that the fixative

depressed the rate of hydrolysis of substrate(decrease in and it also lowered the affinity of enzymes for substrate(increase in KM).Hence ,fixed tissue required two or three times as much substrate to saturate the enzymes,but less substrate was hydrolyzed,as compared to unfixed tissue. 29Dehyration is needed to replace the water in the tissue by alcohol or a substitute,clearing comprises the exchange of alcohol by a reagent with paraffin.30

Many popular mixtures used today are from the original formulas developed many years ago (Evans

and Krajians 1930:

Kristensen 1948:Clayden1952). For practical purposes ,todays laboratories seem to perfer simpler solutions for routine work.

The

purpose of decalcification is to remove calcium salts from mineralized tissue,

resulting in preservation of organic components . Several methods have been employed for decalcification including use of heat, vacuum,electric current and chemical agents . Amongst them, the chemical agents are the commonly used for routine histopathological analysis. The widely used chemical agents for decalcification are either acids, which react with calcium in teeth to form soluble calcium salts or chelating agents which form a complex with calcium.

Acid decalcifiers can be divided into two groups: strong (Inorganic) and weak (organic) acids. As Brain (1966)suggested ,many laboratories keep an acid from each group for either rapid diagnostic

work. Strong inorganic acids

decalcify rapidly,cause tissue swelling,and can seriously damage stainability if used longer than 24-48 hours.Old nitric acid is particularly damaging and should be replaced with fresh

stocks.

Table 1: Mineral acid decalcifiers

Decalcifier

Formula

Comment

Nitric acid

5% in distilled water

Rapid in action, exceeding end-point will impair staining.

Perenyi's fluid (1882)

10% nitric acid 40ml

0.5%

chromic acid 30ml

Absolute alcohol 30

ml

A traditional decalcifier that decalcifies more slowly than aqueous nitric acid.

Quite rapid in action, exceeding end-point will

impair staining.

Hydrochloric acid

5-10% in distilled water

Formalin should be washed from specimen before placing in HCl to avoid the formation of bis-

chloromethyl ether (a carcinogen). Rapid in action, exceeding end-point will impair staining.

Von Ebner's solution

Sodium chloride saturated soln. 50ml

Distilled water 42ml

Hydrochloric acid 8ml

Rapid in action, exceeding end-point will impair staining.

Weak acid decalcifiers

Weak acids such as formic acid are widely used

for decalcification. Formic acid can be used as a simple 10% aqueous solution or combined with formalin or with a buffer.

The salts,sodium chromate(Kristensen1948) or sodium citrate(Evans&Krajian 1930) are added to formic acid solutions making acidic buffers.Buffering is used to counteract the injurious

effects of

the acid, but this ,in addition to low 4-5% formic acid concentration,

results in

increased time needed for complete decalcification. Formic acid is gentler and slower than Hcl or nitric acids

and is suitable for particularly when immunohistochemical staining is needed.Formic acid can still damage tissue ,antigens,and enzyme histochemical staining and should be end point tested.Decalcification is usually complete in 1-10 days,depending on the

acid concentration.

Although it is slower than the strong acid agents it is much gentler in action

and less likely

to interfere with nuclear staining. An example of a proprietary decalcifier based on formic acid is Surgipath's Decalcifier . It also contains formalin and is claimed to fix as well as decalcify and be gentle in action. Other acids such as trichloracetic acid (TCA) have also been used. Picric acid, as a component of some fixatives has weak decalcifying properties

Table2:weak acid decalcifiers

Decalcifier

Formula

Comment

Formic acid

10% in distilled water

A simple effective decalcifier.

Evans and Krajian

Formic acid 25ml

Sodium citrate 10g

Distilled water 75ml

An effective formic acid decalcifier buffered with

citrate.

Kristensen

Formic acid 18ml

Sodium formate 3.5g

Distilled water 82ml

An effective formic acid decalcifier buffered with formate

Gooding and Stewart

Formic acid 5-25ml

40%

formaldehyde 5ml

Distilled water 75

ml

A formic acid decalcifier with added formalin, claimed to fix and decalcify.

Decalcifying agents –

Chelating agents

Chelating agents such as ethylenediaminetetracetic acid (EDTA), work by capturing the calcium ions from the apatite crystal surface, slowly reducing its size.

Because the process is very slow but very gentle (weeks may be required depending on the size of the specimen), but more appropriate for research applications where very high quality morphology is required or particular molecular elements must be preserved for techniques such as IHC, FISH or PCR. It is used at a concentration of approximately 14% as a neutralized solution.

The rate at which EDTA will decalcify is pH dependent.

It is generally used at pH7.0. It works more rapidly at pH10 but some tissue elements can be damaged at alkaline pH.

Table 3: Chelating agents Decalcifier Formula

Comment

Neutral EDTA

EDTA disodium salt 250g

Distilled water 1750ml

Bring to pH 7.0 by adding sodium hydroxide (about 25g will be needed).

Acts slowly but

causes little tissue damage. Conventional stains are largely unaffected.

Factors influencing the rate of decalcification:

Several factors influence the rate of decalcification,and there are ways to speed up or slow down this process.The concentration and

the

volume of the active reagent,including the temperature at which the reaction takes place,are important at all times.

Concentration: The concentration of active agent will affect the rate at which calcium is removed. Published formulations for decalcifying solutions strike a balance between speed and degree of tissue damage. The concentration of active agent will be depleted as it combines with calcium and so it is wise to use a large volume of decalcifier and renew it several times during the decalcification process.

Acid solutions should be end point

tested and changed daily to ensure the decalcifying agent is renewed and that tissues are not left in acids too long or

over-exposed to acids,i.e

overdecalcification.

Temperature: Increased temperature will speed up the decalcification rate but will also increase the rate of tissue damage so must be employed with great care.

Some authors suggested that the microwaves can induce an elevation of the temperature, increasing the decalcification process by decalcified agent diffusion, (Boon&Kok,1998:Balatona&Loget 1989). .(Vongsavanea et al,1990,Tomero et al 1991).At the same time,an increase of the temperature is interesting,but a higher elevation 60 0C is a disaster for the morphological characteristic preservation,(Balatona &Loget:Boon&kok:Tomero et al).

Murayama and his colleagues (1937)reported that

with increasing temperature a gradual decrease in the time necessary for decalcification in nitric acid

was observed.3

Diffusion (a physical process) and chemical-reaction rates are influenced by rise in temperature32 .

The optimal temperature for acid

decalcification has not been determined although Smith (1962) suggested 250 C as the standard temperature but in practice a room temperature(RT) range of 18-300C is acceptable.Conversely,lower temperature decreases reaction rates and

Wallington(1972) suggested that tissues not completely decalcified at the end of a working week could be left in acid at 400C over a weekend.This practice may result in over-decalcification of tissues,even with formic acid.

A better recommendation is to interrupt decalcification by briefly rinsing acid off bone, immersing it in NBF,and resuming decalcification on the next working day. Microwave, sonication and electrolytic methods heat,and must be carefully monitored to prevent excessive temperatures that damage tissue(callis

and Sterchi 1998).

Changes

in temperature at which decalcification occurs also varies the time taken for complete decalcification.

Some authors have suggested that microwaving induces a temperature raise enhancing decalcification by diffusion of the decalcifying solution.Other authors have advocated that the action of microwaving does not increase the diffusion of the decalcified solution but rather promotes a larger disposition of the Ca2+ in this agent due to the formed electromagnetic field.

Agitation:

The effect of agitation on decalcification remains controversial even though it is generally accepted that mechanical agitation influences fluid exchange within as well as around tissues with other reagents, So it would be a logical assumption that agitation speeds up decalcification and studies

specimen and fluid and done attempting to confirm this theory. Russell(1963)

used a tissue processor motor rotating at one revolution per minute and reported the decalcification period was reduced from 5days to 1 day. Others including Clayden(1952) ,Brain (1966),and drury and wallington(1980),repeated or performed similar experiments and failed to find any time reduction.The sonication method vigorously agitates both specimen and fluid,and one study noted cellular debris found on the floor of a container after sonication could possibly

be important

tissue

shaken from the specimen(Callis

and

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Sterchi 1998).

Gentle fluid agitation is achieved by low speed rotation,rocking,stirring or bubbling air into the solution.

Gentle agitation may increase the rate slightly.The effect of agitation on the rate of decalcification has been reported earlier (verdenius and Alma 1958), Birkedal-Hansen 1974.6

Agitation also increases the interaction between the specimen and surrounding decalcifying fluid. They observed that the end-point of decalcification was reached in two-thirds the time when agitation was performed as compared to controls.4 On the other hand,agitation may have contributed to relatively higher damage to tissues than by the conventional technique, resulting in a higher number of artefacts.

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Changes

in temperature at which decalcification occurs also varies the time taken for complete decalcification.

Fluid access:

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The decalcifying fluid should be able to contact with all surfaces of a specimen.

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Samples

can be separated and suspended in the fluid with a thread or placed inside cloth bag tied with thread.

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As with fixation fresh decalcifier should have ready access to all surfaces of the specimen. This will enhance diffusion and penetration into the specimen and facilitate solution, ionization and removal of calcium.38

Haematoxylin and eosin staining:

Embedded tissue was sectioned and dewaxed in xylene - two changes of 10 minutes each. The tissue sections were hydrated in running tap water for 5 minutes. Slides were then transferred to a coplin jar containing hematoxylin stain solution for 5-7 minutes and then placed in running tap water for 2-3 minutes for blueing. Sections were differentiated in acid alcohols (one to two dips). They were water washed for 5 minutes and then transferred to a coplin jar containing eosin stain solution for 30 seconds. They were water washed to remove excess stain and later were dehydrated in absolute alcohol - two changes of one dip each, and were air dried and mounted.39

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DIFFERENT METHODS OF DECALCIFICATION 1. Acid decalcification 2. Ion exchange resin 3. Electrical ionization 4. Chelating methods 5. Surface decalcification

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Decalcification

is a straightforward process but to be successful it requires: A careful preliminary assessment of the specimen Thorough fixation Preparation of slices of reasonable thickness for fixation and processing The choice of a suitable decalcifier with adequate volume, changed regularly A careful determination of the endpoint Thorough processing using a suitable schedule

Methods of

Decalcification

The tissue is cut into small pieces of 3 to 5 mm size. This helps in faster decalcification. The tissue is then suspended in decalcifying medium with waxed thread. The covering of wax on thread prevents from the action of acid on thread. The volume of the decalcifying solution should be 50 to 100 times of the volume of tissue. The decalcification should be checked at the regular interval. Acid Decalcification : This is the most commonly used method. Various acid solutions may be used alone or in combination with a neutralizer. The neutralizer helps in preventing the swelling of the cells. Following are the usually used decalcifying solutions – Aqueous Nitric Acid Nitric acid - 5 ml Distilled water - 100 ml 1. If tissue is left for long time in the solution, the tissue may be damaged. Yellow colour of nitric acid should be removed with urea. But this solution gives good nuclear staining and also rapid action. 2. Nitric Acid Formaldehyde Nitric acid - 10 ml Formaline - 5-10 ml Distilled water upto 100 ml Advantages Rapid action Good nuclear staining Washing with water is not required Formalin protects the tissues from maceration Formic Acid Solution Formic acid - 5 ml Distilled water - 90 ml Formalin - 5 ml In this solution the decalcification is slow. If concentration of formic acid is increased the process is fast but tissue damage is more. 3. Trichloroacitic Acid : This is used for small biopsies. The process of decalcification is slow hence cannot be used for dense bone or big bony pieces. Formal saline (10%) - 95 ml Tricloroacetic acid - 5 gm 4. Ion

Exchange method : In these ammonium salts of sulfonated polystyrene resin is used. The salt is layered on the bottom of the container and formic acid containing fluid is filled. The decalcifying fluid should not contain mineral acid. X-rays can only determine complete decalcification. The advantages of this method are – Faster decalcification Well preserved tissue structures Longer use of resin 5. Electrolytic Method : Formic acid or HCl are used as electrolytic medium. The calcium ions move towards the cathode. Rapid decalcification is achieved but heat produced may damage the cytological details. 6. Chelating Agents : Organic chelating agents absorb metallic ions.

EDTA can bind calcium forming a non-ionized soluble complex. It works best for cancerous bone. This is best method for decalcification of bone marrow biopsies

as it preserves cytological details best. The glycogen of marrow is preserved 7. . EDTA Solution • EDTA - 5.5 gm • Formaline - 100 ml • Distilled water - 900 ml Surface

Decalcification :

The surface layer of paraffin blocks are inverted in 5% HCl for one hour. About top 30 micron is decalcified. It should be washed thoroughly before cutting

METHODS OF DETERMINING OPTIMUM DECALCIFICATION OR ENDPOINT End-Point of Decalcification: The method of end point determination is a tool for quantitative evaluation of decalcification methods and objective assessment of methods- and or tissue-specific factors.40

0: http://www.ihcworld.com/_protocols/histology/decalcification.htm 99%

X-ray (the most accurate way) Chemical testing (accurate) Physical testing (less accurate and potentially damage

of specimen)

Chemical Test:

The following solutions are needed to chemically test for residual calcium. 5%

Ammonium

Hydroxide Stock: Ammonium hydroxide, 28% 5 ml Distilled water 95 ml Mix well 5% Ammonium Oxalate Stock: Ammonium oxalate 5 ml Distilled water 95 ml Mix well Ammonium Hydroxide/Ammonium Oxalate Working Solution: Use equal parts of the 5% ammonium hydroxide solution and the 5% ammonium oxalate solution. Procedure 1.

Insert a pipette into the decalcifying solution containing the specimen. 2. Withdraw approximately 5 ml of the hydrochloric acid/formic acid decalcification solution from under the specimen and place it in a test tube. 3. Add

approximately 10 ml of the ammonium hydroxide/ammonium oxalate working solution, mix well and let stand overnight 4. Decalcification is complete when no precipitate

is

observed on two consecutive days of testing. Repeat this test every two or three days.

Physical Tests

The physical tests include bending the specimen or inserting a pin, razor, or scalpel directly into the tissue.

The disadvantage of inserting a pin, razor, or scalpel is the introduction of tears and pinhole artifacts. Slightly bending the specimen is safer and less disruptive but will not conclusively determine if all calcium salts have been removed. After checking for rigidity, wash thoroughly prior to processing.

METHODOLOGY 80 extracted teeth (N=80) specimen collected from the Department of Oral Surgery,Vivekanandha Dental College for Women were used for the study.The collected teeth were fixed by immersing in 10%formalin solution,immediately after extraction.Inclusion criteria includes, permanent teeth with fully formed roots.Grossly decayed teeth and teeth with structural developmental anomalies were excluded from the study. Among the 80 collected teeth ,40 were single rooted and 40 were multirooted.Then each tooth was labelled ,kept in a container and allowed to undergo decalcification by the following two methods ,namely manual and automated method of decalcification. Manual method of decalcification: In the manual method(GroupA,n=40),among the 40 teeth,20 were single rooted and 20 were multirooted.After fixation in 10% neutral buffered formalin solution,each extracted tooth was placed in a gauze piece and then decalcified in a glass bottle,containing 8% formal nitric acid solution at room temperature. The decalcifying solution was changed every day morning and evening All teeth specimens were checked periodically and the end point of decalcification was determined by radiographic and chemical methods. Fig1:X-rays were taken to determine the decalcification Automated method of decalcification In the automated method (Group B,n=40),among the 40 teeth,20 were single rooted and 20 were multirooted. Each tooth was

0: https://www.slideshare.net/DRBHUVANNAGPAL/24-decalcification-lambert 56%

loosely wrapped in gauze piece and then suspended in the centre of hook of small jar that is filled with the decalcifying fluid . (Automatic decalcifier-Yorco).The volume of

fluid used for decalcification is about 100 times the volume of the tissue. The automated decalcification machine (Yorco) vibrate by using electric current thus tooth in the loosely wrapped gauze may also start to rotate at each vibration. Every day from morning to evening,this process is kept on continuing and the solution was changed every day in the morning freshly.All the teeth specimens undergo decalcification were evaluated periodically and the end point of decalcification was determined by radiographic and chemical methods. After complete decalcification of teeth through manual and automated methods ,the

specimens were washed overnight under running tap water and then dehydrated through a series of alcohol from 70% to 100% alcohol , then were cleared with xylene and embedded in paraffin wax block.Block orientation or embedding is done by using Lshaped mold. . Fig.2:Semi automatic micotome .The blocks were sectioned at 4 µm thickness using Semi-automatic microtome.(Leico RM 2165).They were then stained with Haematoxylin and Eosin(H &E) staining.All the stained specimens,were then blinded and examined under light microscope to evaluate the following parameters namely Dentinal structures,Dentinal staining,Pulp -cellular details(Haematoxylin staining,Eosin staining),Dentin-pulpal integrity and cementum staining. Also,along with the light microscopic observation,

the efficacy of the two methods was assessed based on the following two parameters also. • Time taken for decalcification . • Effect on processing was assessed based on the ease with which the sections could be handled.

The observations were then tabulated,and then statistical analysis was done using chi-square test.P value>0.05 is considered to be statistically significant. Fig 3:Automated decalcifying machine.

RESULTS

Table1: Duration (in days) taken for decalcification

Method of Tooth Decalcification n Mean SD SE t p Duration in days Manual 40 7.40 0.98 0.16 17.23 0.001**

Automated 40 4.10 0.71 0.11 ** Significant at 1 % (Statistically highly significant) Graph1: Duration (in days) taken for decalcification Duration in days Manual Automated 7.4 4.0999999999999996 Method of Tooth Decalcification (Number of days) Mean Table 2: Evaluation of ease of sectioning Ease of sectioning Method of Tooth Decalcification Total Chi square p

Manual Automated N %

n % n % Easy 18 45 21 52.5 39 48.75 1.16 0.559 Difficult 11 27.5 12 30 23 28.75 Very difficult 11 27.5 7 17.5 18 22.50 Total 40 100 40 100 80 100.00

Statistically-not significant Graph 2: Evaluation of ease of sectioning Ease of sectioning Easy Manual Automated 45 52.5 Difficult Manual Automated 27.5 30 Very difficult Manual Automated 27.5 17.5 Method of Tooth Decalcification Ease of sectioning percentage Table 3: Dentinal structure Dentinal structure Method of Tooth Decalcification Total Chi square p

Manual Automated N %

n % n % Clear 23 57.5 15 37.5 38 47.50 3.21 0.073 Not clear 17 42.5 25 62.5 42 52.50 Total 40 100 40 100 80 100.00

Statistically-Not significant Graph 3: Dentinal structure Dentinal structure Clear Manual Automated 57.5 37.5 Not clear Manual Automated 42.5 62.5 Method of Tooth Decalcification Dentinal structure Percentage Table 4: Dentinal staining Dentinal staining Method of Tooth Decalcification Total Chi square p

Manual Automated N %

n % n % Adequate 19 47.5 13 32.5 32 40.00 38.01 0.001** Understained 1 2.5 25 62.5 26 32.50 Overstained 20 50 2 5 22 27.50 Total 40 100 40 100 80 100.00 ** Significant at 1 % (Statistically highly significant) Graph 4: Dentinal staining Dentinal staining Adequate Manual Automated 47.5 32.5 Understained Manual Automated 2.5 62.5 Overstained Manual Automated 50 5 Method of Tooth Decalcification Dentinal staining Percentage Table 5a) Pulp cellular details-Haematoxylin staining

Haematoxylin stain Method of Tooth Decalcification Total Chi square p

Manual Automated N %

n % n % Adequate 20 50 2 5 22 27.50 61.61 0.001** Understained 3 7.5 38 95 41 51.25 Overstained 17 42.5 17 21.25 Total 40 100 40 100 80 100.00 ** Significant at 1 % (Statistically highly significant) Graph 5a) Pulp cellular details-Haematoxylin staining Haematoxylin stain Adequate Manual Automated 50 5 Understained Manual Automated 7.5 95 Overstained Manual Automated 42.5 0 Method of Tooth Decalcification Haematoxylin staining percentage Table 5b) Pulp-cellular details-Eosin staining Eosin stain Method of Tooth Decalcification Total Chi square p

Manual Automated N %

n % n % Adequate 20 50 13 32.5 33 41.25 27.58 0.001** Understained 4 10 25 62.5 29 36.25 Overstained 16 40 2 5 18 22.50 Total 40 100 40 100 80 100.00 ** Significant at 1 % (Statistically highly significant) Graph 5b) Pulp-cellular details-Eosin staining By chisquare Test,P value=0.001 P Eosin stain Adequate Manual Automated 50 32.5 Understained Manual Automated 10 62.5 Overstained Manual Automated 40 5 Method of Tooth Decalcification Table 6: Dentin-pulpal integrity Dentin-pulpal integrity Method of Tooth Decalcification Total Chi square p

Manual Automated N %

n % n % Good 12 30 1 2.5 13 16.25 11.17 0.004** Moderate 15 37.5 22 55 37 46.25 Poor 13 32.5 17 42.5 30 37.50 Total 40 100 40 100 80 100.00 ** Significant at 1 % (Statistically significant) Graph 6: Dentin-pulpal integrity Dentin-pulpal integrity Good Manual Automated 30 2.5 Moderate Manual Automated 37.5 55 Poor Manual Automated 32.5 42.5 Method of Tooth Decalcification Dentin-pulpal integrity percentage Table 7: Cementum staining Cementum staining Method of Tooth Decalcification Total Chi square p

Manual Automated N %

n % n % Adequate 21 52.5 21 26.25 58.46 0.001** Understained 4 10 38 95 42 52.50 Overstained 15 37.5 2 5 17 21.25 Total 40 100 40 100 80 100.00 ** Significant at 1 % (Statistically highly significant) Graph 7: Cementum staining Cementum staining Adequate Manual Automated 52.5 0 Understained Manual Automated 10 95 Overstained Manual Automated 37.5 5 Method of Tooth Decalcification Cementum staininge-Percentage RESULTS In the methods of decalcification, while comparing the manual and automated methods,the difference in the duration of decalcification in days was statistically significant at 1%(P value=0.001) and in the automated method , decalcification duration is shorter(4.1days) than in manual method(7.4 days). The ease of sectioning ,when assessed in both manual and automated method of decalcification by using chi square teswas statistically insignificant, indicating that there is no difference in between the two methods in ease of sectioning. The dentinal structure were observed under microscope as clear or not clear, after decalcification with manual and automated methods ,and it was found that there is no difference in both the methods(Pvalue=0.073)in the observation of the dentinal structures. The dentinal staining was graded adequate,understained and overstained for observation and found that dentinal staining using manual method was more satisfactory than the automated method,where understaining (P value=0.001) was observed more. Similarly,in pulp-cellular details,the haematoxylin staining and eosin staining were graded as adequate, understained ,and overstained and observed.Manual method was better in both Haematoxylin(Pvalue=0.001) staining and Eosin staining(P value=0.001)and the automated method showed understaining in most of the cases. The Dentin-pulp integrity was graded as Good,Moderate,Poor and observed ,where manual method was found to be better(Pvalue= 0.004). The cementum staining was also better using manual method and the automated method presented with more understained specimen(P Value=0.001). DISCUSSION:

Decalcification is the

most

important step in the preparation of oral calcified tissues for microscopic examination. It is important

from two stand points. First, sections of teeth are difficult to obtain without

the

removal of calcium, and second, the effect of the various chemical decalcifiers upon the tissue components differs.

Preparation procedure of hard teeth tissues

starts from its fixation. Choice of a fixing reagent is dependent upon the tissue itself and the purpose for which it is to be preserved.

Fixation with 10%

formalin seems to preserve the pulp tissue and maintain favourable conditions for examination and microscopic analysis of its cell components. 10% buffered formalin is more commonly used because

formalin is powerful as well as rapid in its penetration,

more readily available and may be stored for longer periods.

Thus, here we used 10% neutral buffered formalin to fix the tissues

in our study.1,16

Histological sections are affected by many other variables like processing, cutting technique, staining time, etc. All the above variables were kept consistent

in the present study by the use of standardized techniques and recommended parameters.

Duration Of Decalcification

Decalcification is actually the destruction of an area of tooth by constant bathing in acid.

The time required for decalcification of histologic specimens must be

considered a technical problem of great importance.10 The method of end point determination is a tool for quantitative as well as qualitative evaluation of decalcification methods and should be precisely done to determine the time required for decalcification.11 So, we depended both on radiographic as well as chemical methods for determining the end point of decalcification. Finally, number of days taken for decalcification is calculated after excluding the holidays (if any) in between the decalcification process, and in those holidays, the decalcified specimens were transferred to the water to avoid overdecalcification. Thus, in our study, decalcification is achieved within average of 7.4 days in manual method, which is the longest and the same is 4.1 days in automated method, which is the fastest. With the automated method in our study, there is a constant bathing of tooth specimen by acids on all sides by vibration, which increases the rate of penetration, thereby decreasing the time taken for decalcification. According to Murayama and his colleagues (1937), with increasing temperature, there will be a gradual decrease in the time necessary for decalcification.1

Evaluation Of Ease Of Sectioning

The ease of sectioning obtained by both decalcification methods is graded as easy, difficult and very difficult and its percentage are 45%, 27.5%, 27.5% respectively in manual method. The grades for ease of sectioning obtained by automated method are 52.5% (easy), 30% (difficult), and 17.5% (very difficult). The results of our study show that there is no difference in ease of sectioning between manual and automated methods. This could be because, we have used the same decalcifying agent (8% formal nitric acid) in both the methods and a previous study finds difference in ease of sectioning with the usage of different decalcifying agents.

Dentinal Structure Interpretation

Mineralised dentin is separated from the odontoblast layer by the predentin, a 10 to 40 micrometre thick layer of unmineralised matrix54. Based on the fraying in the dentinal tubules and presence of vapor bubbles, destruction of odontoblast layer, the harmful effects of the decalcifying solutions on dentin are assessed. Acid attacks may also causes zone of decomposed dentin. The Dentinal structure obtained by manual methods was graded as clear and not clear and its percentage are 57.5%,and 42.5% respectively whereas in automated methods the percentage obtained are 37.5% and 62.5%respectively and on comparison, it shows manual methods proved to be better when compared to automated methods.

The destruction of dentin by decalcification and then proteolysis have occured in numerous focal areas leading to necrotic mass of dentin, dentin destructions showing vapour bubbles and dentin fraying. Previous studies suggest

that strong acid decalcification opened up the dentinal tubules quickly and serve as a pathway to the pulp tissue, thus destroying or separating the pulp from dentin.

That is why, rapid decalcification in automated method, cause more

fraying in the dentinal tubules along with more destruction of the odontoblasts architecture.4

Dentinal Staining The dentinal staining obtained by manual methods is graded as adequate, understained and overstained and the percentage obtained respectively are 47.5%, 2.5%,and 50% whereas in automated methods are 32.5% and 62.5% and 5% respectively. When compared to both results, it shows that manual methods proved to be with better efficacy. Understaining is more prominent in the automated method, which may be attributed to the rapid action of the acid impairing the hematoxylin intake.

The most pronounced effect of acid decalcification is the impairment of staining properties.

According to Stevans et al(1990) and callis and sterchi (1998),

strong acids such as nitric acid can decalcify rapidly, but cause serious deterioration of stainability,

which

is dependent on the solution acidity and the time it will take to decalcify. Thus,

we suggest, prolonged waterwash after automated method of decalcification may improve the H&E staining. Pulp Cellular Details – Hematoxylin Stain Pulp cellular details are evaluated and the effect of decalcification methods on pulpal

staining is assessed by intensity of hematoxylin staining of the nuclei and intensity of eosin staining of the cytoplasm. H&E staining is assessed and graded as adequate or over stained or under stained.

Haematoxylin stain obtained by manual methods, graded as adequate, understained, and overstained are with the percentage of 50%, 7.5%, and 42.5% respectively whereas in

automated methods, it was 5% and 95% respectively with no overstaining (0%). When compared to both the methods, the manual methods prove to show better efficacy. Pulp Cellular Details – Eosin Stain The Eosin stain obtained by manual methods are graded as adequate, understained, and overstained and the percentage obtained are 50%, and10 % and 40% respectively whereas in automated methods, the percentage obtained are 32.5% and 62.5% and 5% respectively. Here also, when compared to both the methods, manual method shows better efficacy. Histochemical studies on demineralised material have been limited because of the interference of acid with H&E staining.48-51 The effects on H &E staining after automated method of decalcification can be reduced by improved or prolonged methods of post decalcification acid removal, and adjustment of the staining procedures.43 Dentin Pulp Integrity Dental pulp evaluation is the most sensible part of research protocols followed in assessment of decalcified sections4, because the pulp contains the soft tissue components and is the most affected tissue during decalcification.53 According to Sanjai et al (2012) histological examination of hard dental tissue and pulp is imperative for diagnosis of developmental disorders, pulp pathologies, forensic odontology and research.17 In a perfectly decalcified section, the relation between pulp tissue and dentin should be maintained allowing for simultaneous observation of both structures.26 In our present study,

the dental pulp was examined for the presence of all the four zones of the pulp and the amount of separation of pulp from the surrounding dentin.4 The

Dentin-pulp integrity obtained by manual methods are graded as good, moderate, poor and percentage obtained are 30%, 37.5% and 32.5% respectively whereas in automated methods the results obtained are 2.5%, 55% and 42.5% respectively. When compared to both the methods, manual methods maintains the dentin – pulp integrity better and in the automated method, the percentage of decalcified specimens with good dentin-pulp integrity is very less (2.5%).

Pulp separation from dentinal border and preservation of cellular details is dependent on fixation and the choice of decalcifying agents. Frequently, obtaining

a good histological result for the calcified tissues is not possible without some damage to the soft tissues. On the other hand,

adequate preservation of the soft tissues leaves the specimen incompletely decalcified.

Soft tissues put forth little resistance to the histochemical techniques

and the

lesions affecting hard tissues need intricate, technique sensitive methodology for interpretation and diagnosis.2

According to Fernandes et al, (2007)

it is difficult to fulfil the requirements for simultaneous analysis of mineralised and non-mineralised tissues.

Thus, from our study results, with the usage of strong acids like nitric acid, manual method is better in providing better dentin – pulp integrity than the automated method. Usage of chelating agents like EDTA in the automated machine can improve the soft and hard tissue integrity. Cementum Staining On assessment of cemental staining, the staining is graded as adequate, understained and overstained and the results obtained with manual method are 52.5%, 10% and 37.5% respectively whereas in automated methods, the results obtained are 95% and 5% (understained and overstained) with no adequately stained cementum (0%) in decalcified specimen. The deterioration in cemental staining destruction, more commonly in automated method could

be explained on the basis of lytic effects of the acids.

CONCLUSIONS

Decalcification

of hard tissue specimen is a very technique sensitive method and plays an important role in oral pathology, as decalcification of bone or teeth is done on

a regular day to day basis for histopathology reporting. In our study, 8% formal nitric acid showed the most efficient result as it balances both tissue integrity and time factor

suggesting that it can be used as a stable decalcifying agent for routine histopathological diagnosis.

This study is a step forward in establishing the decalcification dynamics and identifying the method that combines the highest quality of stained sections with the shortest time.

Thus, from our study results, 1) Duration of days taken for decalcification proved to be found better in automated method of decalcification 2) Ease of sectioning shows no difference between manual and automated methods of decalcification 3) Dentinal structure is more clearly seen in manual method and manual method preserves the structural details in a better way than the automated method 4) Dentinal staining is more satisfactory in manual method of decalcification. Understaining is the most commonly seen demerit after automated method of decalcification. 5) Haematoxylin staining in pulpal structures are evaluated to be better in manual method of decalcification 6) Eosin staining in pulpal zones are better in manual method when to compared to automated method. 7) Dentin- pulpal integrity is preserved better in manual method of decalcification than in automated method. 8) Manual method of decalcification proves to be better in cementum staining. Thus, we recommend automated method of decalcification in the histopathological practice for timebound diagnosis and prolonged waterwash after automated method of decalcification may improve the H&E staining of various dental structures. Further studies with different decalcifying agents in larger scale is recommended. 1.

Prasad P, Donoghue M. A comparative study of various decalcification techniques. Indian

Journal of Dental Research. 2013;24(3):302. 2.

Srinivasyaiah, A., Nitin, P. and

Hegde, U. (2016).

Comparison of microwave versus conventional decalcification of teeth using three different decalcifying solutions.

Journal of Laboratory Physicians, 8(2),106. 3. Afreen N.

Decalcification: a simpler and better alternative. journal of dentistry and oral

biosciences. 2011;2(2):10-13. 4.

Mattuella LG, Bento LW, Vier – Pellisser FV, Araiyo FB, Fossati AC.

Comparative analysis of two fixing and two decalcifying solutions for processing of human primary teeth with inactive carious lesion.

Rev

odontocienc. 2007;22:99-105. 5.

Kapila S. Driving the Mineral out Faster: Simple Modifications of the Decalcification Technique.

Journal of clinical and diagnostic

research. 2015: 6. Sanjai K, Patil A, Jayaram S,

Kumarswamy J,

Papaiah L,

Krishnan L.

Evaluation and comparison of decalcification agents on the human teeth.

Journal of Oral and Maxillofacial Pathology. 2012;16(2):222. 7.

Choube A, Astekar M, Choube A, Sapra G, Agarwal A, Rana A. Comparison of decalcifying agents and techniques for human dental tissues. Biotechnic & Histochemistry. 2018;93 (2):99-108. 8.

Verdenius H,

Alma

L. A quantitative study of decalcification methods in histology.

Journal of Clinical Pathology. 1958;11(3):229-236. 9.

Naik R, Pai M, Rai S, Baliga P, Mathai A. Microwave histoprocessing versus conventional histoprocessing. Indian Journal of Pathology and Microbiology. 2008;51(1):12. 10. Kok L, Visser P, Boon M. Histoprocessing with the microwave oven: an update. The Histochemical Journal. 1988;20-20(6-7):323-328. 11.

Waerhaug J.

Decalcification of

Bone and Teeth Under Vacuum- A Rapid Method for Producing Hard Tissue Preparations.

Journal of Dental Research. 1949;28(5):525-526. 12.

Simmelink, J. and

Abrigo, S.. Crystal Morphology and Decalcification Patterns Compared in Rat and Human Enamel and Synthetic Hydroxyapatite. (1989):

Advances in Dental Research, 3(2), pp.241-248. 13. Leong A,

Sormunen R. Microwave Procedures for Electron Microscopy and Resin-Embedded Sections. Micron. 1998;29(5):397-409. 14.

Leong A. Microwaves and Turnaround Times in Histoprocessing. American Journal of Clinical Pathology. 2004;121(4):460-462. 15.

Fernandes, M., Gaio, E., Rosing, C., Oppermann, R. and

Rado, P.

Microscopic qualitative

evaluation of fixation time and decalcification media in rat maxillary periodontium.2007:

Brazilian Oral

Research, 21(2),

pp.134-139. 16.

Zappa J,Bielecka A,Adwent M,Cieslik K,

Sabat D,

Comparison

of different decalcification methods to hard teeth tissues morphological analysis.

Dent.Med.Probl.2005:42(1):21-26. 17.

Gupta S. Qualitative Histological Evaluation of Hard and Soft Tissue Components of Human Permanent Teeth Using Various Decalcifying Agents - A Comparative Study. Journal of clinical and diagnostic research, 2014: 8(9), 69-72. 18.

Warshawsky H, moore G. a technique for the fixation and decalcification of rat incisors for electron microscopy .

Journal of Histochemistry & Cytochemistry. 1967;15(9):542-549. 19. Coleman E, Desalva S. Rapid Decalcification for Histochemistry. Journal of Dental Research. 1966;45(4):1237-1237. 20.

Birkedal-Hansen K, Kinetics of acid demineralization in histologic technique. Journal of Histochemistry & Cytochemistry. 1974;22(6):434-441. 21. Cook S, Ezra-cohn H. A comparison of methods for decalcifying bone. Journal of Histochemistry & Cytochemistry. 1962;10(5):560-563. 22.

Bourque W, Gross M, Hall B. A histological processing technique that preserves the integrity of calcified tissues (bone, enamel), yolky amphibian embryos, and growth factor antigens in skeletal tissue.

Journal of Histochemistry & Cytochemistry. 1993;41(9):1429-1434. 23. Laboux O, Dion N, Arana-Chavez V, Ste-Marie L, Nanci A. Microwave Irradiation of Ethanol-fixed Bone Improves Preservation, Reduces Processing Time, and Allows Both Light and Electron Microscopy on the Same Sample. Journal of Histochemistry & Cytochemistry. 2004;52(10):1267-1275. 24. Goland P, Scheiman-Tagger E, Engel M. Enamel Preservation during Decalcification Following Fixation by Some Reactive Halogen Compounds. Journal

of Dental Research. 1965;44(2):342-349. 25.

Callis GM,

Bancroft JD. Theory and Practice of Histological Techniques. 6th ed. Edinburgh: Churchill Livingstone; 2008;

p. 338-360. 26.

Smith C. Effect of glutaraldehyde and decalcifying agents on acid phosphomonoester hydrolase activity in the enamel organ of the rat incisor: a biochemical study comparing enamel organ with liver.

Journal of Histochemistry & Cytochemistry. 1980;28(7):689-699. 27.

Kok L, Boon M. Physics of microwave technology in histochemistry. The Histochemical Journal. 1990;22:381-388. 28.

Pitol D, Caetano F, Lunardi L.

Microwave-induced fast decalcification of rat bone for electron microscopic analysis: an ultrastructural and cytochemical study.

Brazilian Dental Journal. 2007;18(2):153-157. 29.

Morse A.

Formic Acid-Sodium Citrate Decalcification and Butyl Alcohol Dehydration of Teeth and Bones for Sectioning in

Paraffin.

Journal of Dental Research. 1945;24(34):143-153. 30.

0: http://www.ijmdent.com/article.asp?issn=2229-6360;year=2017;volume=7;issue=2;spage=94;epage=100;aulast=Kadashetti 81%

Babu

T, Malathi N, Magesh K. A comparative study on microwave and routine tissue processing. Indian

Journal

of

Dental Research. 2011;22(1):50. 31.

Selvig K. Ultrastructural changes in human dentine exposed to a weak acid.

Archives of Oral Biology. 1968;13(7):719-734. 32.

Lillie R, Laskey A, Greco J, Burtner H, Jones P. Decalcification of Bone in Relation to Staining

and Phosphatase Technics.

American Journal of Clinical Pathology. 1951;21(8):711-722. 33. Schajowicz F, Cabrini R. The effect of acids (decalcifying solutions) and enzymes on the histochemical behavior of bone and cartilage. Journal of Histochemistry & Cytochemistry. 1955;3(2):122-129. 34. Thorell B, Wilton À. The nucleotide metabolism of the dentine cells under normal conditions and in avitaminosis c. Acta Pathologica Microbiologica Scandinavica. 2009;22(6):593-602. 35. Wislocki G, Sognnaes R. Histochemical reactions of normal teeth. American Journal of Anatomy. 1950;87(2):239-275. 36.

Crespi R, Grossi S. A Method for Histological Examination of Undecalcified Teeth. Biotechnic & Histochemistry. 1992;67(4):202-206. 37.

Uma K, Chandavarkar V, Sangeetha R.

Comparison of routine decalcification methods with microwave decalcification of bone and teeth.

Journal of

Oral and Maxillofacial Pathology. 2013;17(3):386. 38.

Mawhinney W, Richardson E, Malcolm A.

Control of rapid nitric acid decalcification. Journal of Clinical Pathology. 1984;37(12):1409-1413. 39.

Reith E, Boyde A. The pyroantimonate reaction and transcellular transport of calcium in rat molar enamel organs. Histochemistry. 1985;83(6):539-543. 40. Massa L, Bradaschia-Correa V, Arana-Chavez V. Immunocytochemical Study of Amelogenin Deposition during the Early Odontogenesis of Molars in Alendronate-treated Newborn Rats. Journal of Histochemistry & Cytochemistry. 2006;54(6):713-725. 41.

Vongsavan N, Matthews B, Harrison G. Decalcification of teeth in a microwave oven.

The Histochemical Journal. 1990;22:377-380. 42.

Mardfin

D, James V. Effect of Nitric Acid and Chelation Demineralization on Various Stains of Histochemical Nature.

Journal

of Dental Research. 1957;36(5):759-764. 43. Morales A, Nassiri M, Kanhoush R, Vincek V, Nadji M. Experience With an Automated Microwave-Assisted Rapid Tissue Processing Method. American Journal of Clinical Pathology. 2004;121(4):528-536. 44. Kayser K, Stute H, Lubcke J, Wazinski U. Rapid microwave fixation?a comparative morphometric study. The Histochemical Journal. 1988;20-20(6-7):347-352. 45.

Boon M, Kok L, Ouwerkerk-noordam E. Microwave-stimulated diffusion for fast processing of tissue: reduced dehydrating, clearing, and impregnating times. Histopathology. 1986;10 (3):303-309. 46.

Donath K, Breuner G. A method for the study of undecalcified bones and teeth with attached soft tissues. The Sage-Schliff (sawing and grinding) Technique. Journal of Oral Pathology and Medicine. 1982;11(4):318-326. 47.

Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 5

th ed.

Missouri: Harcourt Publishers; 2002. 48. Culling’s CF, Allison RT, Histological Technique Processing. 4th ed. London : Butterworths

and Co-publishers; 1985. p. 51-77 . 49.

Culling CF, Allison RT, Barr WT. Cellular Pathology Technique. 4th ed. London: Butterworths; 1984;

p. 408-30. 50.

Stan, PH. Calcified tissue. In: Boon ME, Kok LP (Editors)MicrowaveCookBook of Pathology. Coulomb Press Leynden: Leinden;1988:264-266. 51. Wagenaar F, Kok GL, Broekhuijsen-Davies JM, Pol JMARapid

cold fixation of tissue samples by microwave irradiation goes it uses in electron microscopy. Histochem J, 1993;25:719-725. 52.

Carletons HM,and Drury RAB,Histological techniques,3rd ed.,Oxford university press,New york1957. 53. Cook DJ. In Cellular pathology: Introduction to techniques and applications. 2nd ed. Oxfordshire: Scion; 2006. Processing and microtomy; p. 31–36.

Duration in days Manual Automated 7.4 4.0999999999999996 Method of Tooth Decalcification (Number of days) Mean Eosin stain Adequate Manual Automated 50 32.5 Understained Manual Automated 10 62.5 Overstained Manual Automated 40 5 Method of Tooth Decalcification Haematoxylin stain Adequate Manual Automated 50 5 Understained Manual Automated 7.5 95 Overstained Manual Automated 42.5 0 Method of Tooth Decalcification Haematoxylin staining percentage Dentinal staining Adequate Manual Automated 47.5 32.5 Understained Manual Automated 2.5 62.5 Overstained Manual Automated 50 5 Method of Tooth Decalcification Dentinal staining Percentage Cementum staining Adequate Manual Automated 52.5 0 Understained Manual Automated 10 95 Overstained Manual Automated 37.5 5 Method of Tooth Decalcification Cementum staininge-Percentage Dentinal structure Clear Manual Automated 57.5 37.5 Not clear Manual Automated 42.5 62.5 Method of Tooth Decalcification Dentinal structure Percentage Ease of sectioning Easy Manual Automated 45 52.5 Difficult Manual Automated 27.5 30 Very difficult Manual Automated 27.5 17.5 Method of Tooth Decalcification Ease of sectioning percentage Dentin-pulpal integrity Good Manual Automated 30 2.5 Moderate Manual Automated 37.5 55 Poor Manual Automated 32.5 42.5 Method of Tooth Decalcification Dentin-pulpal integrity percentage

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As with fixation fresh decalcifier should have ready access to all surfaces of the specimen. This will enhance diffusion and penetration into the specimen and facilitate solution, ionization and removal of calcium.38	As with fixation fresh decalcifier should have ready access to all surfaces of the specimen. This will enhance diffusion and penetration into the specimen and facilitate solution, ionization and removal of calcium.

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7	100%	7: https://basicmedicalkey.com/bone-6/	100%
Sterchi 1998). Gentle fluid agitation is achieved by low speed rotation,rocking,stirring or bubbling air into the solution.		Sterchi 1998). Gentle fluid agitation is achieved by low-speed rotation, rocking, stirring, or bubbling air into the solution.	

9	96%	9: https://basicmedicalkey.com/bone-6/	96%
The decalcifying fluid should be able to contact with all surfaces of a specimen.		The decalcifying fluid should be able to make contact with all surfaces of a specimen	

10	92%	10: https://basicmedicalkey.com/bone-6/	92%
Samples can be separated and suspended in the fluid with a thread or placed inside cloth bag tied with thread.		samples can be separated and suspended in the fluid with a thread or placed inside cloth bags tied with thread	

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14 99%

X-ray (the most accurate way) Chemical testing (accurate) Physical testing (less accurate and potentially damage

of specimen)

Chemical Test:

The following solutions are needed to chemically test for residual calcium. 5%

Ammonium

Hydroxide Stock: Ammonium hydroxide, 28% 5 ml Distilled water 95 ml Mix well 5% Ammonium Oxalate Stock: Ammonium oxalate 5 ml Distilled water 95 ml Mix well Ammonium Hydroxide/ Ammonium Oxalate Working Solution: Use equal parts of the 5% ammonium hydroxide solution and the 5% ammonium oxalate solution. Procedure 1.

Insert a pipette into the decalcifying solution containing the specimen. 2. Withdraw approximately 5 ml of the hydrochloric acid/formic acid decalcification solution from under the specimen and place it in a test tube. 3. Add

14: http://www.ihcworld.com/_protocols/histology/decalcification.htm 99%

X-ray (the most accurate way) Chemical testing (accurate) Physical testing (less accurate and potentially damage of specimen) Chemical Test: The following solutions are needed to chemically test for residual calcium. 5% Ammonium Hydroxide Stock: Ammonium hydroxide, 28% ---------- 5 ml Distilled water ---------- 95 ml Mix well 5% Ammonium Oxalate Stock: Ammonium oxalate ---------- 5 ml Distilled water ---------- 95 ml Mix well Ammonium Hydroxide/Ammonium Oxalate Working Solution: Use equal parts of the 5% ammonium hydroxide solution and the 5% ammonium oxalate solution. Procedure: • Insert a pipette into the decalcifying solution containing the specimen. • Withdraw approximately 5 ml of the hydrochloric acid/formic acid decalcification solution from under the specimen and place it in a test tube. • Add approximately 10 ml of the ammonium hydroxice/ammonium oxalate working solution, mix well and let stand overnight. • Decalcification is complete when no precipitate is observed on two consecutive days of testing. Repeat this test every two or three days. Physical Tests: The physical tests include bending the specimen or inserting a pin, razor, or scalpel directly into the tissue. The disadvantage of inserting a pin, razor, or scalpel is the introduction of tears and

approximately 10 ml of the ammonium hydroxide/ammonium oxalate working solution, mix well and let stand overnight 4. Decalcification is complete when no precipitate

is

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Physical Tests

The physical tests include bending the specimen or inserting a pin, razor, or scalpel directly into the tissue.

The disadvantage of inserting a pin, razor, or scalpel is the introduction of tears and pinhole artifacts. Slightly bending the specimen is safer and less disruptive but will not conclusively determine if all calcium salts have been removed. After checking for rigidity, wash thoroughly prior to processing.

pinhole artifacts. Slightly bending the specimen is safer and less disruptive but will not conclusively determine if all calcium salts have been removed. After checking for rigidity, wash thoroughly prior to processing.

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6 100%

So, an ideal decalcifying agent should be fast, be good and do good.

6: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3424938/ 100%

So, an ideal decalcifying agent should

• Be fast; • Be good and; • Do good.

8 100%

Changes

in temperature at which decalcification occurs also varies the time taken for complete decalcification.

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Changes in temperature at which decalcification occurs also varies the time taken for complete decalcification.

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3 100%

The goal of decalcification is to remove calcium salts from the mineralized tissues,

3: http://aimdrjournal.com/pdf/Vol2Issue3_17_OA_Shahid_2_3_25.pdf 100%

The goal of decalcification is to remove calcium salts from the mineralized tissues,

4 100%

them for further sectioning of the histological specimen.

Any acid, even if properly buffered, affects tissue stability. These effects depend on the solution’s acidity and duration of the decalcification process.

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them for further sectioning of the histological specimen. Any acid, even if properly buffered, affects tissue stability. These effects depend on the solution’s acidity and duration of the decalcification process.

5 96%

The

factors influencing the speed of

decalcification include decalcifying solution concentration, temperature, stirring and tissue suspension. Decalcification is performed by chemical solutions, which employ acids (acids may be

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The factors influencing the speed of decalcification include decalcifying solution concentration, temperature, stirring and tissue suspension. [2] Decalcification is performed by chemical solutions, which employ acids (acids may be divided into strong and weak acids or chelates. [3-5] It is ought to be emphasized that fixative agents that contain acid in their composition, such as formalin (that contains formic acid), may also be able to act as

divided into strong and weak acids or chelates). It is thought to

be emphasized that fixative agents that contain acid in their composition, such as formalin (that contains formic acid), may also

be

able to act as decalcifying agents if the acid component is not neutralized.

Quicker decalcification is

decalcifying agents if the acid component is not neutralized. [3] Decalcification is

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1 97%

Decalcification is a process of complete

removal of calcium salt from mineralized tissues

like bone, teeth and other calcified tissues.

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Decalcification is a process of complete removal of calcium salt from mineralized tissues like bone and teeth and other calcified tissues.

16 41%

the efficacy of the two methods was assessed based on the following two parameters also. • Time taken for decalcification . • Effect on processing was assessed based on the ease with which the sections could be handled.

16: http://www.ijdr.in/article.asp?issn=0970-9290;year=2013;volume=24;issue=3;spage=302;epage=308;aulast=Prasad 41%

The efficacy of different decalcifying agents used in the study was evaluated on the basis of following parameters:

• Time taken for decalcification based on chemical estimation of end-point. • Effect on processing was assessed based on ribbon formation, scoring/splitting of sections during cutting and the ease with which the sections could be handled. •

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15	56%

loosely wrapped in gauze piece and then suspended in the centre of hook of small jar that is filled with the decalcifying fluid . (Automatic decalcifier-Yorco).The volume of

15: https://www.slideshare.net/DRBHUVANNAGPAL/24-decalcification-lambert	56%

loosely wrapped in gauze and then suspended in the center of a large jar that is filled with the decalcifying fluid of choice. About 100 times the volume of

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Decalcification

is a straightforward process but to be successful it requires: A careful preliminary assessment of the specimen Thorough fixation Preparation of slices of reasonable thickness for fixation and processing The choice of a suitable decalcifier with adequate volume, changed regularly A careful determination of the endpoint Thorough processing using a suitable schedule

Methods of

Decalcification

The tissue is cut into small pieces of 3 to 5 mm size. This helps in faster decalcification. The tissue is then suspended in decalcifying medium with waxed thread. The covering of wax on thread prevents from the action of acid on thread. The volume of the decalcifying solution should be 50 to 100 times of the volume of tissue. The decalcification should be checked at the regular interval. Acid Decalcification : This is the most commonly used method. Various acid solutions may be used alone or in combination with a neutralizer. The neutralizer helps in preventing the swelling of the cells. Following are the usually used decalcifying solutions – Aqueous Nitric Acid Nitric acid - 5 ml Distilled water - 100 ml 1. If tissue is left for long time in the solution, the tissue may be damaged. Yellow colour of nitric acid

13: https://nios.ac.in/media/documents/dmlt/HC/Lesson-06.pdf 96%

Decalcification is a straightforward process but to be successful it requires: z A careful preliminary assessment of the specimen z Thorough fixation z Preparation of slices of reasonable thickness for fixation and processing z The choice of a suitable decalcifier with adequate volume, changed regularly z A careful determination of the endpoint z Thorough processing using a suitable schedule Methods of Decalcification The tissue is cut into small pieces of 3 to 5 mm size. This helps in faster decalcification. The tissue is then suspended in decalcifying medium with waxed thread. The covering of wax on thread prevents from the action of acid on thread. The volume of the decalcifying solution should be 50 to 100 times of the volume of tissue. The decalcification should be checked at the regular interval. Acid Decalcification – This is the most commonly used method. Various acid solutions may be used alone or in combination with a neutralizer. The neutralizer helps in preventing the swelling of the cells. Following are the usually used decalcifying solutions - 1. Aqueous Nitric Acid- Nitric acid - 5 ml Distilled water - 100 ml If tissue is left for long time in the solution, the tissue may be damaged. Yellow colour of nitric acid should be removed with urea. But this solution gives good nuclear staining and also rapid action. 2. Nitric Acid Formaldehyde Nitric acid - 10 ml Formaline - 5-10 ml

should be removed with urea. But this solution gives good nuclear staining and also rapid action. 2. Nitric Acid Formaldehyde Nitric acid - 10 ml Formaline - 5-10 ml Distilled water upto 100 ml Advantages Rapid action Good nuclear staining Washing with water is not required Formalin protects the tissues from maceration Formic Acid Solution Formic acid - 5 ml Distilled water - 90 ml Formalin - 5 ml In this solution the decalcification is slow. If concentration of formic acid is increased the process is fast but tissue damage is more. 3. Trichloroacitic Acid : This is used for small biopsies. The process of decalcification is slow hence cannot be used for dense bone or big bony pieces. Formal saline (10%) - 95 ml Tricloroacetic acid - 5 gm 4. Ion Exchange method : In these ammonium salts of sulfonated polystyrene resin is used. The salt is layered on the bottom of the container and formic acid containing fluid is filled. The decalcifying fluid should not contain mineral acid. X-rays can only determine complete decalcification. The advantages of this method are – Faster decalcification Well preserved tissue structures Longer use of resin 5. Electrolytic Method : Formic acid or HCl are used as electrolytic medium. The calcium ions move towards the cathode. Rapid decalcification is achieved but heat produced may damage the cytological details. 6. Chelating Agents : Organic chelating agents absorb metallic ions.

EDTA can bind calcium forming a non-ionized soluble complex. It works best for cancerous bone. This is best method for decalcification of bone marrow biopsies

HISTOLOGY AND CYTOLOGY MODULE Decalcification Histology and Cytology 30 Notes Distilled water upto 100 ml Advantages z Rapid action z Good nuclear staining z Washing with water is not required z Formalin protects the tissues from maceration 3. Formic Acid Solution Formic acid - 5 ml Distilled water - 90 ml Formalin - 5 ml In this solution the decalcification is slow. If concentration of formic acid is increased the process is fast but tissue damage is more. 4. Trichloroacitic Acid - This is used for small biopsies. The process of decalcification is slow hence cannot be used for dense bone or big bony pieces. Formal saline (10%) - 95 ml Tricloroacitic acid - 5 gm Ion Exchange method – In these ammonium salts of sulfonated polystyrene resin is used. The salt is layered on the bottom of the container and formic acid containing fluid is filled. The decalcifying fluid should not contain mineral acid. X-rays can only determine complete decalcification. The advantages of this method are - z Faster decalcification z Well preserved tissue structures z Longer use of resin Electrolytic Method – Formic acid or HCl are used as electrolytic medium. The calcium ions move towards the cathode. Rapid decalcification is achieved but heat produced may damage the cytological details. Chelating Agents – Organic chelating agents absorb metallic ions. EDTA can bind calcium forming a non-ionized soluble complex. It works best for cancerous bone. This is best method for decalcification of bone marrow biopsies as it preserves cytological details best. The glycogen of marrow is preserved. EDTA Solution EDTA - 5.5 gm Formaline - 100 ml Distilled water - 900 ml

as it preserves cytological details best. The glycogen of marrow is preserved 7. . EDTA Solution • EDTA - 5.5 gm • Formaline - 100 ml • Distilled water - 900 ml Surface

Decalcification :

The surface layer of paraffin blocks are inverted in 5% HCl for one hour. About top 30 micron is decalcified. It should be washed thoroughly before cutting

31 Decalcification HISTOLOGY AND CYTOLOGY MODULE Histology and Cytology Notes Surface Decalcification – The surface layer of paraffin blocks are inverted in 5% HCl for one hour. About top 30 micron is decalcified. It should be washed thoroughly before cutting.

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In routine histopathology,

decalcification of teeth is an essential and important step during tissue processing,

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DIFFERENT METHODS OF DECALCIFICATION 1. Acid decalcification 2. Ion exchange resin 3. Electrical ionization 4. Chelating methods 5. Surface decalcification

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DIFFERENT METHODS OF DECALCIFICATION 1. Acid decalcification 2. Ion exchange resin 28 2 3. Electrical ionization 4. Chelating methods 5. Surface decalcification

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Dr. K. Gayathri.

LIST OF FIGURES

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LIST OF ABBREVIATIONS

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S.NO		**Abbreviations**
1.	**H**	**Haematoxylin**
2.	**E**	**Eosin**
3.	**HNO_3**	**Nitric oxide**
4.	**HCL**	**Hydrochloric acid**
5.	**EDTA**	**Ethyelene diamine tetra acetic acid.**
6	**ml**	**Milli litre**
7.	**gm**	**grams**
8.	**NH_4OH**	**Ammonium hydroxide**
9.	**$_{Ca}2+$**	**Calcium**
10.	**mm**	**Milli metres**

S.NO		**Abbreviations**
11.	**RT**	**Room Temperature**
12.	**0C**	**Celsius**
13.	**CO_2**	**Carbon dioxide**
14.	**KM**	**Concentration of substrate**
15.	**IHC**	**Immunohistochemistry**
16.	**FISH**	**Flourescent in situ hybridisation**
17.	**PCR**	**Polymerase chain reaction**
18.	**NBF**	**Neutral buffered formalin**
19.	**$C_2H_8N_2O_4$**	**Ammonium oxalate**

CONTENTS

CONTENTS

INTRODUCTION

INTRODUCTION

Decalcification is a process of complete removal of calcium salt from mineralized tissues like bone, teeth and other calcified tissues.[1] In routine histopathology, decalcification of teeth is an essential and important step during tissue processing, and plays an important role in histological diagnostic techniques.[2] Various acids or chelating agents are used for decalcification in the laboratories for microscopic examination of hard tissues. The goal of decalcification is to remove calcium salts from the mineralized tissues, while preserving the organic portions and preparing them for further sectioning of the histological specimen. Any acid, even if properly buffered, affects the tissue stability. These effects depend on the solution's acidity and duration of the decalcification process. The factors influencing the speed of decalcification include decalcifying solution concentration, temperature, stirring and tissue suspension. Decalcification is performed by chemical solutions, which employ acids (acids may be divided into strong and weak acids or chelates). It is thought to be emphasized that fixative agents that contain acid in their composition, such as formalin (that contains formic acid), may also be able to act as decalcifying agents if the acid component is not neutralized.[3] Quicker decalcification is essential for faster diagnosis of hard tissue pathology.[4] So, an ideal decalcifying agent should be fast, be good and do good.[5] Apart from properties of decalcifying agents, the method of decalcification also affects the outcome of the decalcified specimen. Automated decalcifying instruments are available,which enhances the process by constant rotation and stirring of the specimen and maintaining the temperature.Thus, the aim of our study is to compare automated and manual tissue processing methods, to know the best method for studying the decalcified specimens.

AIM AND OBJECTIVES

AIM OF THE STUDY

1) To compare between manual and auto mated methods of decalcification.

OBJECTIVES OF THE STUDY

1) To decalcify extracted teeth specimen by manual method of decalcification (Group A).
2) To decalcify extracted teeth specimen by automated method of decalcification (Group B).

3) To compare group A and group B for

1)Number of days taken for decalcification.

2)Ease of Sectioning.

4) To compare the H &E stained histological sections of Group A and Group B for

1)Dentinal structures.

2)Dentinal staining.

3)Pulp-cellular details-Haematoxylin staining.

4)Pulp cellular details-Eosin staining.

5)Dentin –pulpal integrity.

6)Cementum staining.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

According to Drury and Wallington (1980), strong acids, weak acids, chelating agents have been used to remove calcium ions from hard tissues. Although decalcification by strong acids is rapid, it damages tissue and affects tissue stainability. Decalcification with weak acids, such as formic acid can preserves tissue details, but is time consuming. Chelating agents, such as EDTA, do not damage tissues, but they require a long time (Callis 2008). According to Verdenius and Alma (1958).Decalcification can be doneby applying heat, agitation, vacuum and electric current . Microwave energy (Sangeetha et al2013) and ultrasonic cleaners (Hatta et al. 2014) also have been used to accelerate decalcification.[6]

The speed and quality of histological processes including the decalcification of teeth and bone, can be better enhanced if they are carried out in a microwave oven. (Boon&kok,1988). Balaton and Loget (1989) have also reported that the decalcification of bone is accelerated about ten times compared with that ambient temperature.[7] Verdenius and Alma in 1958 compared other methods of decalcification and their modifications namely, different temperatures, different acids, use of electric current, Vaccum, and physical movement. The following methods have been described in the literature.

HEAT

Heat is a method to hasten the decalcification process.[4] A chemical process is accelerated two to three times for every 100^0 C. of temperature, and Murayama, Suzuki, and Itoh (1937) revealed that with increased temperature the decalcification process takes place within an even smaller duration of time.[8] Heat increased the

diffusion of reaction products, which decreased the decalcification time (verdenius and Alma 1958).In their study, Verdenius and Alma(1958) showed that heat is also known to accelerate decalcification as it raises the diffusion rate and increases the chemical reaction rate. They also observed that the time used for decalcification decreased as temperature was increased from 13^{o}C to 25^{o}C to 40^{o}C . Similarly, intermittent heating to 45^{o}C in a hot air oven showed fastest decalcification of teeth specimens.[4] Heat reduces the liquid viscositythereby, speeding the rate of diffusion of reagents in to and out of the tissue.[9] Agitation was slower than heating by 1 day, but it can preserve the Pulp cellular details .[4]

MOVEMENT

In this method the possibility of interaction between the object and the surrounding decalcifying fluid is increased.[8]Diffusion is a key factor in histoprocessing. Boon et al., (1986) describe how microwaves increases diffusion of reagents into the tissue, and out of it. As explained in some detail by Kok (1986), the rise in temperature increases diffusion rates. The key formula for diffusion is = 2Dt. Here x = the net distance covered by a particle in solution in a particular direction, t = time period during which the diffusion process takes place, D = the diffusion constant, characteristic of solute and solvent. The brackets signify 'average', hence in words the formula expresses: the average squared distance covered by a particle in solution is proportional to the diffusion time. This indicate the need to maintain biopsies thickness small; three times as thick means nine times as long for comparable effects. Note that width and length of the biopsy hardly matter. To obtain the same findings in a 10 times shorter time, one must increase D by a factor of 10.

Now D is highly temperature dependent. Furthermore, it is advantageous to work with small molecules.[10]

VACUUM

The process of decalcification can be characterized as follows by creating a vacuum: Insoluble calciurn-salts+acid o-soluble calcium salts+CO2.

The carbon dioxide, rapidly extracted, would cause disturbance in the balance of the chemical equilibrium, and, according to Le Chatelier, it can result in an acceleration of the reaction. Waerhaug (1949), however, showed that there will be a more rapid contact between the specimen and the surrounding fluid, the CO2 bubbles can being rapidly removed. Waerhaug (1949), Frank and Deluzarche (1950),and Engelbreth-Holm and Plum (1951) also showed a considerable shortening of the time of decalcification. According to Molenaar (1957) only, the decalcification process only slightly accelerated.[8] In a study by waerhaug,bone and teeth were decalcified rapidly under vaccum and the time taken for decalcification was reduced to one –tenth.[5]

ELECTRIC CURRENT

Under the influence of an electric field ,the Ca ions liberated by the decalcifying fluid will be removed more rapidly. Richman, Gelfand, and Hill (1947), Ducey and Shippy (1950), Dolan (1951), and Scheliga(1952) revealed that the process of decalcification is greatly accelerated, whereas the stainability will remain unchanged. According to various group of investigators (Lillie, Laskey, Greco, Burtner, and Jones, (1951), the increased speed of reaction was arised to result only from the rise in temperature due to the passage of the electric current.

These results were confirmed by Molenaar in 1958.[8] In 1937 Murayame Suzuki and, Itoh revealed that with increased temperature for the process of decalcification actually takes place within a shorter time. Goncalves&Oliverio used an electric decalcification techniques, with alternate chain,increasing the decalcification velocity.According to these authors,this process promotes molecule shaking,resulting in an increase of the decalcification process.[3] The study conducted by Nylen et al. (1963) showed that increased enamel calcification was due to an increase in crystal size. However, there is disagreement on the morphology and decalcification pattern of rat enamel crystals (Warshawsky, 1987a,b; Warshawsky et al., 1987).[11] The use of Microwaves as a method to fix tissue for neurochemical analysis is well established (Stavinoha et al., 1970; Schneider et al., 1982) .[12] In 1995, Boon et al reported that it was possible to produce significant acceleration of tissue processing by introducing microwaves . In 1998, Visinoni et al, followed up with a description of the first microwave tissue processor that completed processing in between 30 and 120 minutes, depending on the thickness of the specimen .[13]

In 2003, Correa et al. reported that the decalcification process causes important morphological alterations in the tissues such as vacuolation ,edema, and ruptures not contributable to the pathologic condition.[14] Zappa J et al., (2005) compared the decalcification of 288 carious and non-carious teeth with 10% EDTA, 10% EDTA-TRIS, and electrolytic decalcification with Romeis fluid. They have concluded that Romeis fluid was the best for routine histopathology diagnosis and EDTA was the best for educational and research purposes where time is not a constraint.In 2011 NadafA,Madhura S, Radhika M B,Parimala ,Sudhakara M. suggest that Decalcify thirty teeth in 5%nitric acid, 10% nitric acid, 10% nitric acid-10%

formalin and10% nitric acid – 20% formalin at 55°C. Decalcified teeth of Group containing 10 % HNO3 and 20% formalin proved to be faster among the three groups while maintaining perfect tissue details.[15]Sanjai K, Kumarswamy J, Patil A, Papaiah L, Jayaram S, Krishnan L(2012) have compared the rate of decalcification of six decalcifying agents and their effect on staining of dental tissues.They also studied 5% nitric acid, Pereneyi's fluid, formal nitric acid, 5% trichloroacetic acid, EDTA, and 10% formic acid

They reported that EDTA was the slowest but preserved soft tissue integrity and staining characteristics very well, while 5% nitric acid was fastest but least considerate to the tooth structure[5]. Prasad P and Donoghue M(2013) have conducted a study on six DAs: 10% formal nitric acid,8% formal nitric acid, Pereneyi's fluid and EDTA, on posterior mandible of rat. They found that both 8% and 10% formal nitric acid gave quick decalcification and good tissue results. Apart from the properties of decalcifying agents,the method of decalcification can also affects the outcome of the decalcified specimen.[1]

Decalcification is the commonly employed technique in histopathology laboratories as a part of calcified tissue preparation for the microscopic examination.[2] Chemical agents are commonly used for routine decalcification procedures, though some agents adversely damage the tissue integrity and staining properties.[16]Tooth decalcification is a time consuming process. It takes days to weeks and tissue preservation structure depends on the quality of the demineralization process[2].The degree of decalcification was determined by the lack of resistance to cutting the specimen with a razor blade. The time required for decalcification differs directly with the size of specimen.[17] when enzymes histochemical localization is to

be done on decalcified hard tissues, it is important to perform the decalcification in the shortest period time possible in order to preserve as much of the enzyme as possible in their original state.[18]Histologic studies of mature hard tissues , teeth and bone have been conducted for the most part with formalin fixation and acid demineralization.[19-23] The diffusion plays an important role and that the decalcification progress is significantly affected by the rate of diffusion of decalcifying solution in the specimen.[24] Research has been done in the introduction of new decalcifying as well as modification of known decalcifying agents to meet good decalcifying agent criteria, which should ensure calcium removal completely at a good speed with minimal damage to cells and tissues.[2] Solid tissues need to be processed, to preserve their structures and eventually impregnated with an appropriate hardening substance to permit making thin slices suitable for microscopic evaluation.Inorganic component of teeth can be studied in ground sections, but decalcification is required to study the organic components . Moreover, the pulpal soft tissue which otherwise is difficult to appreciate in the ground sections, can be easily assessed in decalcified sections. Decalcification is routinely used technique in most histopathological laboratories for the microscopic examination of calcified tissues. Decalcification may be regarded as any treatment which destroys the inorganic phase, with the removal of the essential element calcium, in such a way so as to leave the organic portion sufficiently intact to be handled and sectioned by normal methods.[25] Correlation of cytological and morphological features with immunophenotyping is difficult.[26]A number of histological methods have been adapted over the many years to analyze normal and pathological features of calcified tissues.[27] For almost 100 years, the steps followed to prepare tissues for microscopic review have remained practically unchanged. Decalcification was checked by serial roentgenograms at

various stages of the process.[28] The choice of decalcifier is affected by four interdependent factors;urgency of the case,degree of mineralisation,extent of the investigation and staining techniques. Gray (1954) listed over 50 different mixtures.Manyof these mixtures were developed for special purposes with one used as a fixing and dehydrating reagents.e.g,Buffer salts,chromic acid,formalin or Ethanol, intended to counteract the swelling effects of acids on tissues.The process of dehydrating,clearing and impregnating agent required to obtain paraffin sections is time consuming. All three steps are based on diffusion,and the aim is to replace one reagent by another.[29]Lineweaver-burk plots revealed that the fixative depressed the rate of hydrolysis of substrate(decrease in and it also lowered the affinity of enzymes for substrate(increase in KM).Hence ,fixed tissue required two or three times as much substrate to saturate the enzymes,but less substrate was hydrolyzed,as compared to unfixed tissue.[29]Dehyration is needed to replace the water in the tissue by alcohol or a substitute,clearing comprises the exchange of alcohol by a reagent with paraffin.[30] Many popular mixtures used today are from the original formulas developed many years ago(Evans and Krajians 1930:Kristensen 1948:Clayden1952).

For practical purposes ,todays laboratories seem to perfer simpler solutions for routine work.The purpose of decalcification is to remove calcium salts from mineralized tissue, resulting in preservation of organic components . Several methods have been employed for decalcification including use of heat, vacuum,electric current and chemical agents . Amongst them, the chemical agents are the commonly used for routine histopathological analysis. The widely used chemical agents for decalcification are either acids, which react with calcium in teeth to form soluble calcium salts or chelating agents which form a complex with calcium.Acid decalcifiers can be divided into two groups:strong (Inorganic) and weak(organic)

acids.As Brain (1966)suggested ,many laboratories keep an acid from each group for either rapid diagnostic work. Strong inorganic acids decalcify rapidly,cause tissue swelling,and can seriously damage stainability if used longer than 24-48 hours.Old nitric acid is particularly damaging and should be replaced with fresh stocks.

Table 1: Mineral acid decalcifiers

Decalcifier	Formula	Comment
Nitric acid	5% in distilled water	Rapid in action, exceeding end-point will impair staining.
Perenyi's fluid (1882)	10% nitric acid 40ml 0.5% chromic acid 30ml Absolute alcohol 30ml	A traditional decalcifier that decalcifies more slowly than aqueous nitric acid. Quite rapid in action, exceeding end-point will impair staining.
Hydrochloric acid	5-10% in distilled water	Formalin should be washed from specimen before placing in HCl to avoid the formation of bis-chloromethyl ether (a carcinogen). Rapid in action, exceeding end-point will impair staining.
Von Ebner's solution	Sodium chloride saturated soln. 50ml Distilled water 42ml Hydrochloric acid 8ml	Rapid in action, exceeding end-point will impair staining.

WEAK ACID DECALCIFIERS

Weak acids such as formic acid are widely used for decalcification. Formic acid can be used as a simple 10% aqueous solution or combined with formalin or with a buffer. The salts, sodium chromate (Kristensen1948) or sodium citrate (Evans&Krajian 1930) are added to formic acid solutions making acidic buffers. Buffering is used to counteract the injurious effects of the acid, but this, in addition to low 4-5% formic acid concentration, results in increased time needed for complete decalcification. Formic acid is gentler and slower than Hcl or nitric acids and is suitable for particularly when immune histochemical staining is needed. Formic acid can still damage tissue, antigens and enzyme histochemical staining and should be end point tested. Decalcification is usually complete in 1-10 days, depending on the acid concentration. Although it is slower than the strong acid agents it is much gentler in action and less likely to interfere with nuclear staining. An example of a proprietary decalcifier based on formic acid is Surgipath's Decalcifier. It also contains formalin and is claimed to fix as well as decalcify and be gentle in action. Other acids such as trichloracetic acid (TCA) have also been used. Picric acid, as a component of some fixatives has weak decalcifying properties.

Table2:weak acid decalcifiers

Decalcifier	Formula	Comment
Formic acid	10% in distilled water	A simple effective decalcifier.
Evans and Krajian	Formic acid 25ml Sodium citrate 10g Distilled water 75ml	An effective formic acid decalcifier buffered with citrate.
Kristensen	Formic acid 18ml Sodium formate 3.5g Distilled water 82ml	An effective formic acid decalcifier buffered with formate
Gooding and Stewart	Formic acid 5-25ml 40%formaldehyde 5ml Distilled water 75ml	A formic acid decalcifier with added formalin, claimed to fix and decalcify.

DECALCIFYING AGENTS – CHELATING AGENTS

Chelating agents such as ethylenediaminetetracetic acid (EDTA), work by capturing the calcium ions from the apatite crystal surface, slowly reducing its size. Because the process is very slow but very gentle (weeks may be required depending on the size of the specimen), but more appropriate for research applications where very high quality morphology is required or particular molecular elements must be preserved for techniques such as IHC, FISH or PCR. It is used at a concentration of approximately 14% as a neutralized solution. The rate at which EDTA will decalcify is pH dependent. It is generally used at pH7.0. It works more rapidly at pH10 but some tissue elements can be damaged at alkaline pH.

Table 3: Chelating agents

Decalcifier	Formula	Comment
Neutral EDTA	EDTA disodium salt 250g Distilled water 1750ml Bring to pH 7.0 by adding sodium hydroxide (about 25g will be needed).	Acts slowly but causes little tissue damage. Conventional stains are largely unaffected.

FACTORS INFLUENCING THE RATE OF DECALCIFICATION

Several factors influence the rate of decalcification, and there are ways to speed up or slow down this process. The concentration and the volume of the active reagent, including the temperature at which the reaction takes place, are important at all times.

CONCENTRATION

The concentration of active agent will affect the rate at which calcium is removed. Published formulations for decalcifying solutions strike a balance between speed and degree of tissue damage. It must be remembered that the concentration of active agent will be depleted as it combines with calcium and so it is wise to use a large volume of decalcifier and renew it several times during the decalcification process. Ideally ,acid solutions should be end point tested and changed daily to ensure the decalcifying agent is renewed and that tissues are not left in acids too long or over-exposed to acids, i.e .over decalcification.

TEMPERATURE

Increased temperature will speed up the decalcification rate but will also increase the rate of tissue damage so must be employed with great care. Some authors suggested that the microwaves can induce an elevation of the temperature, increasing the decalcification process by decalcified agent diffusion, (Boon & Kok, 1998: Balatona & Loget 1989), (Vongsavanea et al,1990,Tomero et al 1991).At the same time, an increase of the temperature is interesting, but a higher elevation 60^0C is a disaster for the morphological characteristic preservation, (Balatona & Loget: Boon & kok: Tomero et al). Murayama and his colleagues (1937)reported that with increasing temperature a gradual decrease in the time necessary for decalcification in nitric acid was observed.[3] Diffusion (a physical process) and chemical-reaction rates are influenced by rise in temperature[32] . The optimal temperature for acid decalcification has not been determined although Smith (1962) suggested 25^0 C as the standard temperature but in practice a room temperature(RT) range of 18-30^0C is acceptable. Conversely, lower temperature decreases reaction rates and Wallington(1972) suggested that tissues not completely decalcified at the end of a working week could be left in acid at 40^0C over a weekend. This practice may result in over-decalcification of tissues, even with formic acid. A better recommendation is to interrupt decalcification by briefly rinsing acid off bone, immersing it in NBF, and resuming decalcification on the next working day. Microwave, sonication and electrolytic methods heat, and must be carefully monitored to prevent excessive temperatures that damage tissue (callis and Sterchi1998). Changes in temperature at which decalcification occurs also varies the time taken for complete decalcification. Some authors have suggested that microwaving induces a temperature raise enhancing decalcification by diffusion of

the decalcifying solution. Other authors have advocated that the action of microwaving does not increase the diffusion of the decalcified solution but rather promotes a larger disposition of the Ca^{2+} in this agent due to the formed electromagnetic field.[33-37]

AGITATION

The effect of agitation on decalcification remains controversial even though it is generally accepted that mechanical agitation influences fluid exchange within as well as around tissues with other reagents. Therefore, it would be a logical assumption that agitation speeds up decalcification and studies specimen and fluid and done attempting to confirm this theory. Russell(1963) used a tissue processor motor rotating at one revolution per minute and reported the decalcification period was reduced from 5 days to 1 day. Others including Clayden (1952), Brain(1966), and drury and wallington (1980),repeated or performed similar experiments and failed to find any time reduction. The sonication method vigorously agitates both specimen and fluid, and one study noted cellular debris found on the floor of a container after sonication could possibly be important tissue shaken from the specimen(Callis and Sterchi 1998).Gentle fluid agitation is achieved by low speed rotation, rocking, stirring or bubbling air into the solution**.** Gentle agitation may increase the rate slightly. The effect of agitation on the rate of decalcification has been reported earlier(verdenius and Alma 1958), Birkedal-Hansen 1974.[6]

Agitation also increases the interaction between the specimen and surrounding decalcifying fluid. They observed that the end-point of decalcification was reached in two-thirds the time when agitation was performed as compared to controls.[4] On the other hand, agitation may have contributed to relatively higher damage to tissues than

by the conventional technique, resulting in a higher number of artefacts. Changes in temperature at which decalcification occurs also varies the time taken for complete decalcification.

FLUID ACCESS

The decalcifying fluid should be able to contact with all surfaces of a specimen. Samplescan be separated and suspended in the fluid with a thread or placed inside cloth bag tied with thread. As with fixation fresh decalcifier should have ready access to all surfaces of the specimen. This will enhance diffusion and penetration into the specimen and facilitate solution, ionization and removal of calcium.[37]

HAEMATOXYLIN AND EOSIN STAINING

Embedded tissue was sectioned and dewaxed in xylene - two changes of 10 minutes each. The tissue sections were hydrated in running tap water for 5 minutes. Slides were then transferred to a coplin jar containing hematoxylin stain solution for 5-7 minutes and then placed in running tap water for 2-3 minutes for blueing. Sections were differentiated in acid alcohols (one to two dips). They were water washed for 5 minutes and then transferred to a coplin jar containing eosin stain solution for 30 seconds. They were water washed to remove excess stain and later were dehydrated in absolute alcohol - two changes of one dip each, and were air dried and mounted.[38]

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DIFFERENT METHODS OF DECALCIFICATION

1. Acid decalcification
2. Ion exchange resin
3. Electrical ionization
4. Chelating methods
5. Surface decalcification

Decalcification is a straightforward process but to be successful it requires:

- A careful preliminary assessment of the specimen
- Thorough fixation
- Preparation of slices of reasonable thickness for fixation and processing
- The choice of a suitable decalcifier with adequate volume, changed regularly
- A careful determination of the endpoint
- Thorough processing using a suitable schedule

METHODS OF DECALCIFICATION

The tissue is cut into small pieces of 3 to 5 mm size. This helps in faster decalcification. The tissue is then suspended in decalcifying medium with waxed thread. The covering of wax on thread prevents from the action of acid on thread. The volume of the decalcifying solution should be 50 to 100 times of the volume of tissue. The decalcification should be checked at the regular interval.

ACID DECALCIFICATION

This is the most commonly used method. Various acid solutions may be used alone or in combination with a neutralizer. The neutralizer helps in preventing the swelling of the cells.

Following are the usually used decalcifying solutions –

Aqueous Nitric Acid

Nitric acid - 5 ml

Distilled water - 100 ml

1. If tissue is left for long time in the solution, the tissue may be damaged. Yellow colour of nitric acid should be removed with urea. But this solution gives good nuclear staining and also rapid action.

2. Nitric Acid Formaldehyde

   Nitric acid - 10 ml

   Formalin - 5-10 ml

   Distilled water upto 100 ml

ADVANTAGES

- Rapid action
- Good nuclear staining
- Washing with water is not required
- Formalin protects the tissues from maceration

Formic Acid Solution

Formic acid - 5 ml

Distilled water - 90 ml

Formalin - 5 ml

In this solution the decalcification is slow. If concentration of formic acid is increased the process is fast but tissue damage is more.

3. TRICHLOROACETIC ACID :

- This is used for small biopsies.
- The process of decalcification is slow hence cannot be used for dense bone or big bone pieces.

Formal saline (10%) - 95 ml

Tricloroacetic acid - 5 gm

4. ION EXCHANGE METHOD :

In these ammonium salts of sulfonated polystyrene resin is used. The salt is layered on the bottom of the container and formic acid containing fluid is filled. The decalcifying fluid should not contain mineral acid. X-rays can only determine complete decalcification.

The advantages of this method are

- Faster decalcification
- Well preserved tissue structures
- Longer use of resin

5. ELECTROLYTIC METHOD:

Formic acid or HCl are used as electrolytic medium. The calcium ions move towards the cathode. Rapid decalcification is achieved but heat produced may damage the cytological details.

6. CHELATING AGENTS:

Organic chelating agents absorb metallic ions. EDTA can bind calcium forming a non-ionized soluble complex. It works best for cancerous bone. This is best method for decalcification of bone marrow biopsies as it preserves cytological details best. The glycogen of marrow is preserved.

7. EDTA SOLUTION:

- EDTA - 5.5 gm
- Formaline - 100 ml
- Distilled water - 900 ml

SURFACE DECALCIFICATION :

The surface layer of paraffin blocks are inverted in 5% HCl for one hour. About top 30 micron is decalcified. It should be washed thoroughly before cutting.

METHODS OF DETERMINING OPTIMUM DECALCIFICATION OR END-POINT OF DECALCIFICATION

The method of end point determination is a tool for quantitative evaluation of decalcification methods and objective assessment of methods and or tissue-specific factors.[40]

- X-ray (the most accurate way)
- Chemical testing (accurate)
- Physical testing (less accurate and potentially damage of specimen)

CHEMICAL TEST

The following solutions are needed to chemically test for residual calcium.

5% Ammonium Hydroxide Stock:

Ammonium hydroxide, 28% 5 ml

Distilled water 95 ml

Mix well

5% Ammonium Oxalate Stock:

Ammonium oxalate 5 ml

Distilled water 95 ml

Mix well

Ammonium Hydroxide/Ammonium Oxalate Working Solution:

Use equal parts of the 5% ammonium hydroxide solution and the 5% ammonium oxalate solution.

PROCEDURE

1. Insert a pipette into the decalcifying solution containing the specimen.
2. Withdraw approximately 5 ml of the hydrochloric acid/formic acid decalcification solution from under the specimen and place it in a test tube.
3. Add approximately 10 ml of the ammonium hydroxide/ammonium oxalate working solution, mix well and let stand overnight.
4. Decalcification is complete when no precipitate is observed on two consecutive days of testing. Repeat this test every two or three days.

PHYSICAL TESTS

The physical tests include bending the specimen or inserting a pin, razor, or scalpel directly into the tissue. The disadvantage of inserting a pin, razor, or scalpel is the introduction of tears and pin hole artifacts. Slightly bending the specimen is safer and less disruptive but will not conclusively determine if all calcium salts have been removed. After checking for rigidity, wash thoroughly prior to processing.

MATERIALS AND METHODS

METHODOLOGY

80 extracted teeth (N=80) specimen collected from the Department of Oral Surgery, Vivekanandha Dental College for Women were used for the study. The collected teeth were fixed by immersing in 10% formalin solution, immediately after extraction. Inclusion criteria includes, permanent teeth with fully formed roots. Grossly decayed teeth and teeth with structural developmental anomalies were excluded from the study. Among the 80 collected teeth, 40 were single rooted and 40 were multirooted. Then each tooth was labelled, kept in a container and allowed to undergo decalcification by the following two methods, namely manual and automated method of decalcification.

MANUAL METHOD OF DECALCIFICATION:

In the manual method (Group A, n=40), among the 40 teeth, 20 were single rooted and 20 were multirooted. After fixation in 10% neutral buffered formalin solution, each extracted tooth was placed in a gauze piece and then decalcified in a glass bottle, containing 8% formal nitric acid solution at room temperature. The decalcifying solution was changed every day morning and evening. All teeth specimens were checked periodically and the end point of decalcification was determined by radiographic and chemical methods.

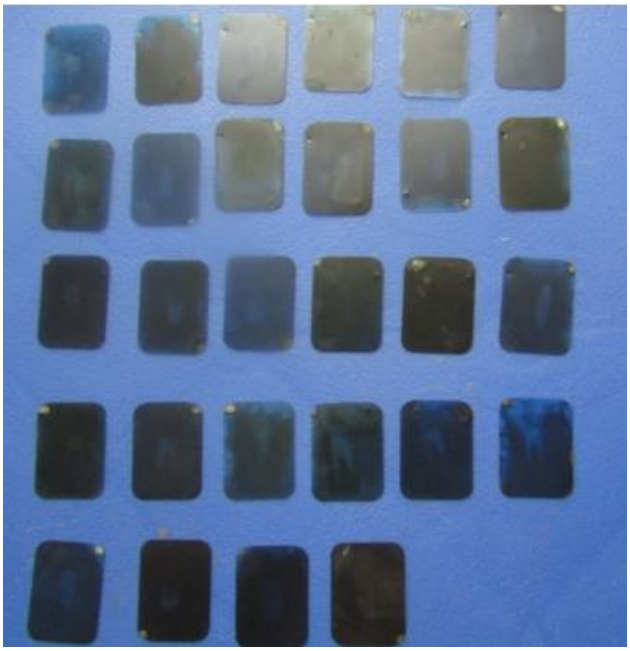


Fig1: X-rays taken to determine the end point of decalcification

AUTOMATED METHOD OF DECALCIFICATION

In the automated method (Group B, n=40), among the 40 teeth, 20 were single rooted and 20 were multirooted. Each tooth was loosely wrapped in gauze piece and then suspended in the centre of hook of small jar that is filled with the decalcifying fluid (Automatic decalcifier-Yorco).The volume of fluid used for decalcification is more than 10 times the volume of the tissue. The automated decalcification machine (Yorco) vibrate by using electric current thus tooth in the loosely wrapped gauze may also start to rotate at each vibration. Every day from morning to evening, this process is kept on continuing and the solution was changed every day in the morning freshly. All the teeth specimens undergoing decalcification were evaluated periodically and the end point of decalcification was determined by radiographic and chemical methods.

After complete decalcification of teeth through manual and automated methods ,the specimens were washed overnight under running tap water and then dehydrated through a series of alcohol from 70% to 100% alcohol , then were cleared with xylene and embedded in paraffin wax block. Block orientation or embedding is done by using L shaped mold.

Fig.2: Semi automatic microtome

The blocks were sectioned at 4 µm thickness using Semi-automatic microtome. (LeicoRM 2165). They were then stained with Haematoxylin and Eosin (H &E) staining. All the stained specimens, were then blinded and examined under light microscope to evaluate the following parameters namely Dentinal structures, Dentinal staining, Pulp-cellular details (Haematoxylin staining, Eosin staining), Dentin-pulpal integrity and cementum staining. Also, along with the light microscopic observation, the efficacy of the two methods was assessed based on the following two parameters also.

- Time taken for decalcification.
- Effect on processing was assessed based on the ease with which the sections could be handled.

The observations were then tabulated, and then statistical analysis was done using chi-square test. P value<0.05 is considered to be statistically significant.

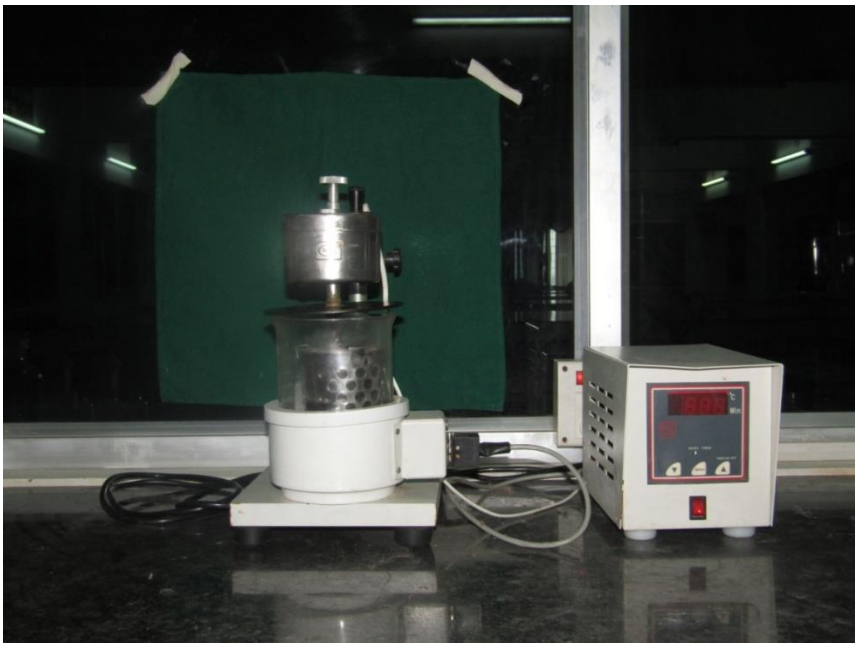


Fig 3: Automated decalcifying machine.

RESULTS

RESULTS

Table1:Duration (in days) taken for decalcification

	Method of Tooth Decalcification	n	Mean	SD	SE	t	p
Duration in days	Manual	40	7.40	0.98	0.16	17.23	0.001**
	Automated	40	4.10	0.71	0.11		

**** Significant at 1 %**
(Statistically highly significant)

Graph1:Duration (in days) taken for decalcification

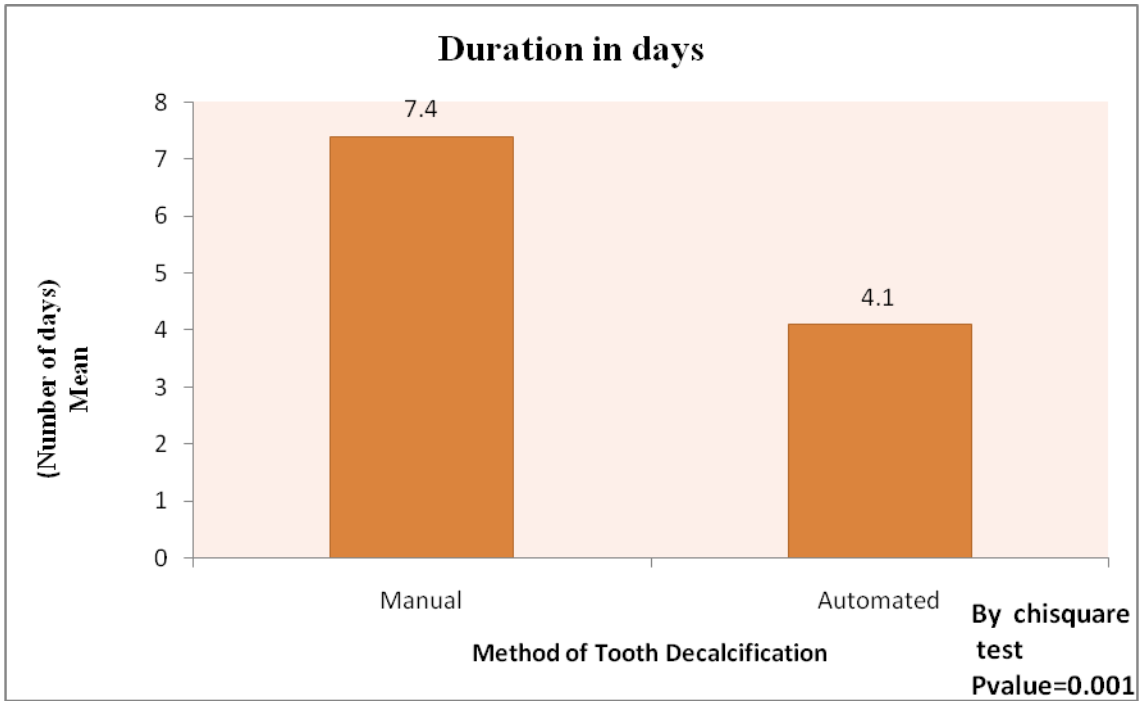


Table 2: Evaluation of ease of sectioning

Ease of sectioning	Method of Tooth Decalcification				Total		Chi square	p
	Manual		Automated		N	%		
	n	%	n	%				
Easy	18	45	21	52.5	39	48.75	1.16	0.559
Difficult	11	27.5	12	30	23	28.75		
Very difficult	11	27.5	7	17.5	18	22.50		
Total	40	100	40	100	80	100.00		

Statistically-not significant

Graph 2: Evaluation of ease of sectioning

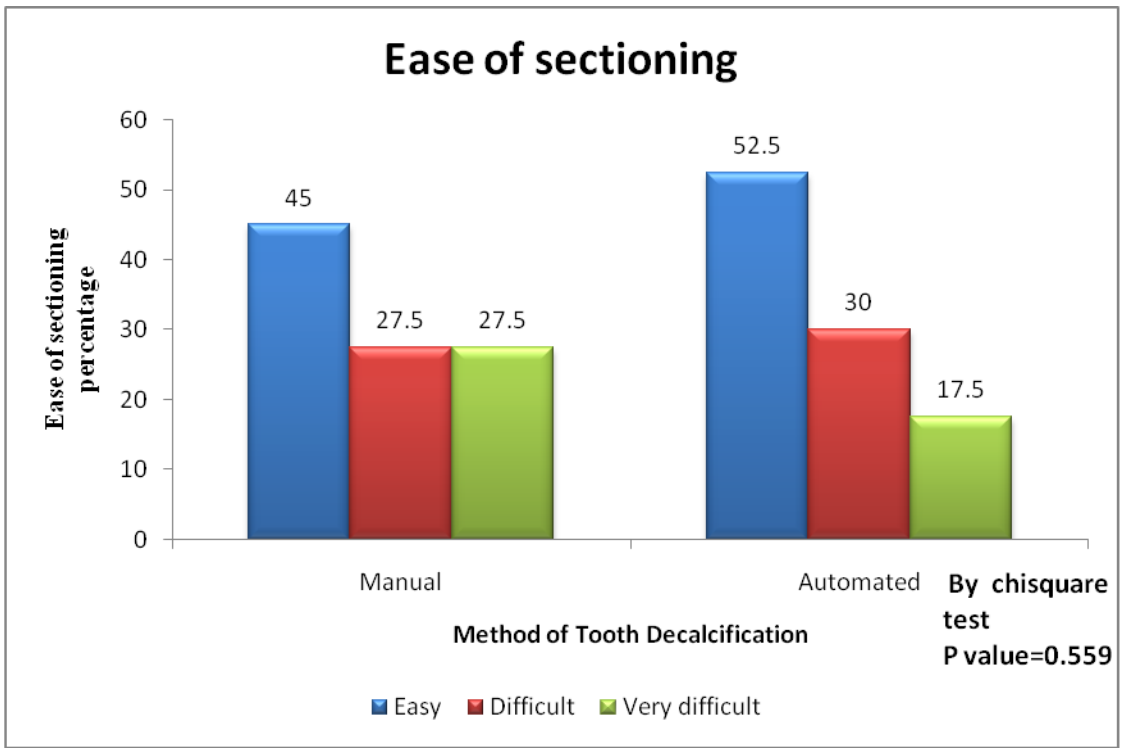


Table 3: Dentinal structure

Dentinal structure	Method of Tooth Decalcification				Total		Chi square	p
	Manual		Automated		N	%		
	n	%	n	%				
Clear	23	57.5	15	37.5	38	47.50	3.21	0.073
Not clear	17	42.5	25	62.5	42	52.50		
Total	40	100	40	100	80	100.00		

Statistically-Not significant

Graph 3: Dentinal structure

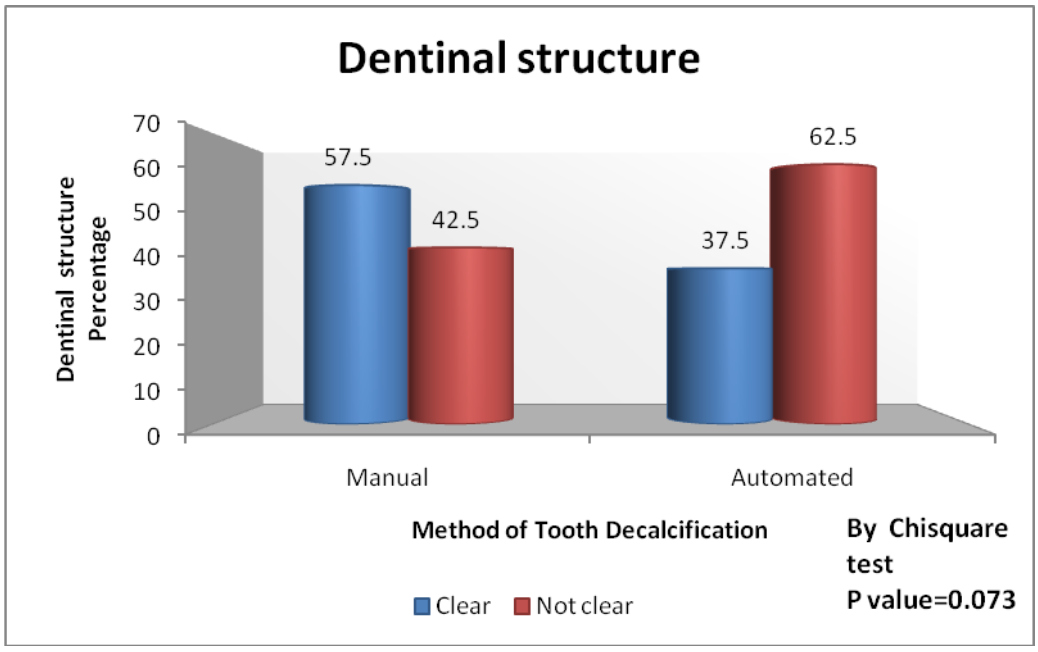


Table 4: Dentinal staining

Dentinal staining	Method of Tooth Decalcification				Total		Chi square	p
	Manual		Automated		N	%		
	n	%	n	%				
Adequate	19	47.5	13	32.5	32	40.00	38.01	0.001**
Understained	1	2.5	25	62.5	26	32.50		
Overstained	20	50	2	5	22	27.50		
Total	40	100	40	100	80	100.00		

**** Significant at 1 %**
(Statistically highly significant)

Graph 4: Dentinal staining

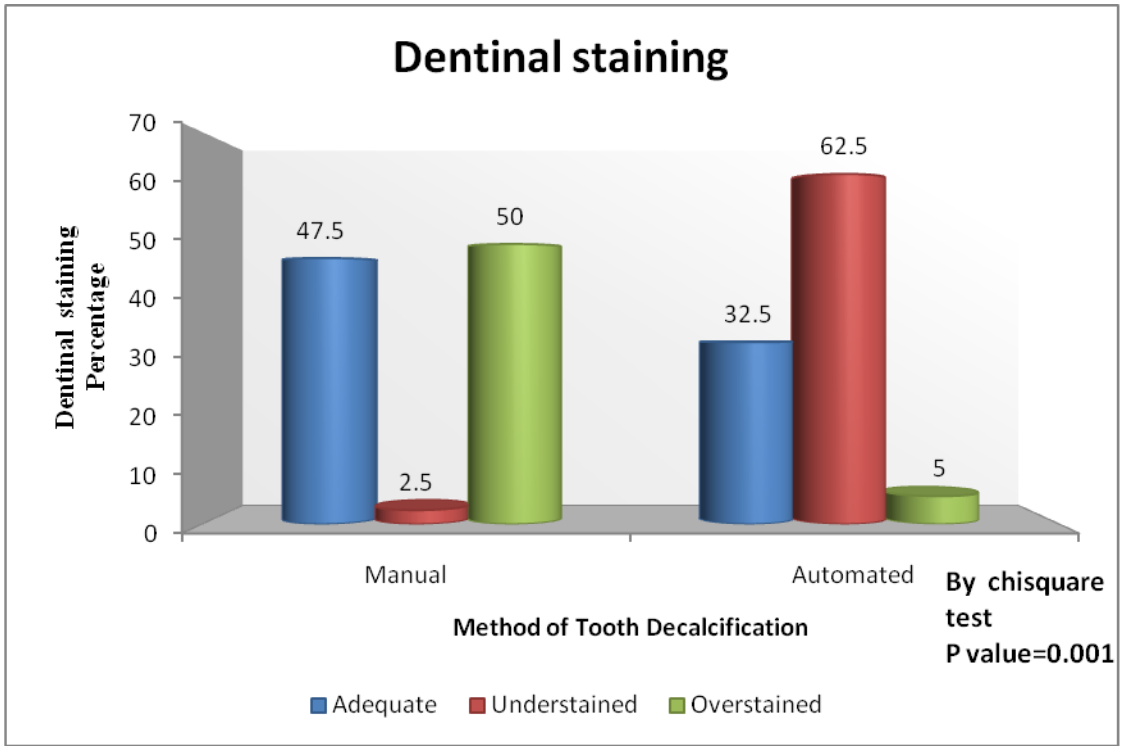


Table 5a) Pulp cellular details-Haematoxylin staining

Haematoxylin stain	Method of Tooth Decalcification				Total		Chi square	p
	Manual		Automated		N	%		
	n	%	n	%				
Adequate	20	50	2	5	22	27.50	61.61	0.001**
Understained	3	7.5	38	95	41	51.25		
Overstained	17	42.5			17	21.25		
Total	40	100	40	100	80	100.00		

**** Significant at 1 %**
(Statistically highly significant)

Graph 5a) Pulp cellular details-Haematoxylin staining

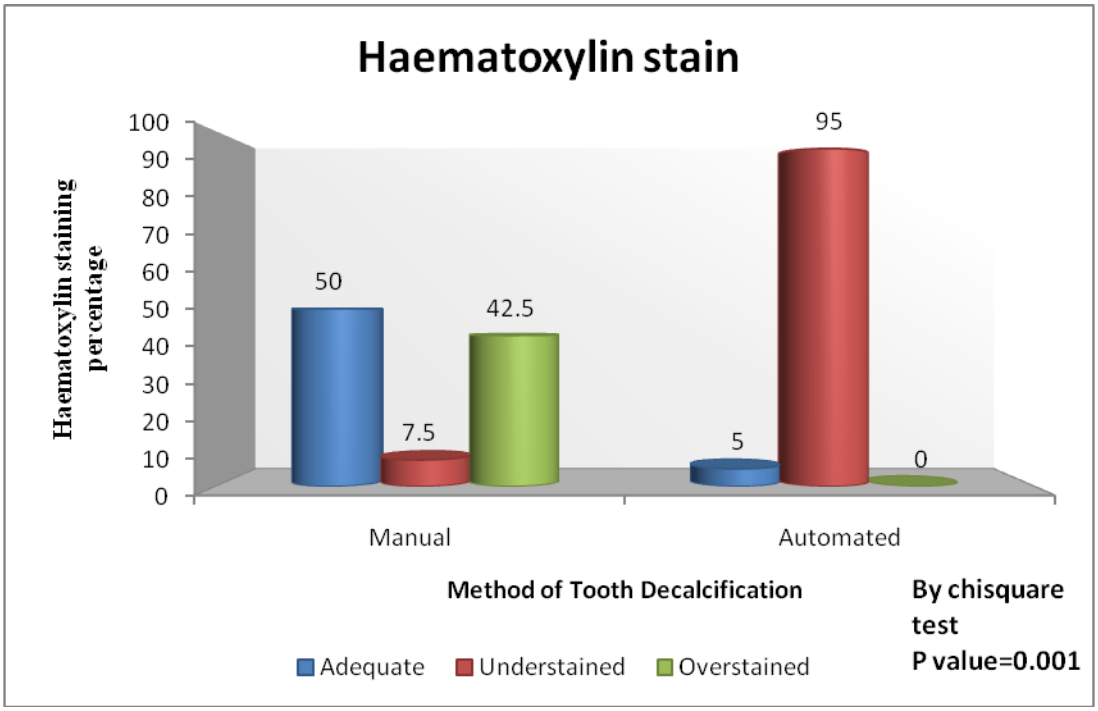


Table 5b) Pulp-cellular details-Eosin staining

Eosin stain	Method of Tooth Decalcification				Total		Chi square	p
	Manual		Automated		N	%		
	n	%	n	%				
Adequate	20	50	13	32.5	33	41.25	27.58	0.001**
Understained	4	10	25	62.5	29	36.25		
Overstained	16	40	2	5	18	22.50		
Total	40	100	40	100	80	100.00		

**** Significant at 1 %**
(Statistically highly significant)

Graph 5b) Pulp-cellular details-Eosin staining

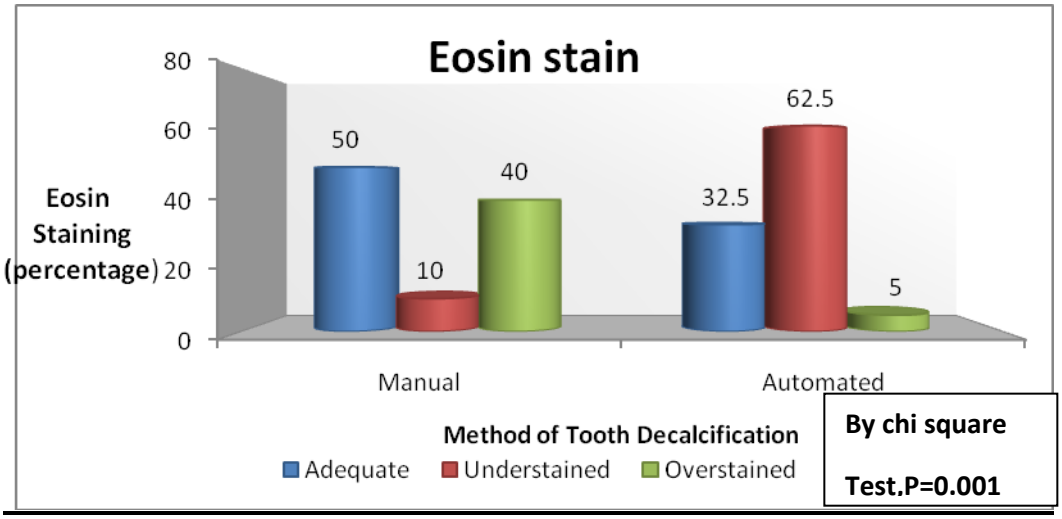


Table 6: Dentin-pulpal integrity

Dentin-pulpal integrity	Method of Tooth Decalcification				Total		Chi square	p
	Manual		Automated		N	%		
	n	%	n	%				
Good	12	30	1	2.5	13	16.25	11.17	0.004**
Moderate	15	37.5	22	55	37	46.25		
Poor	13	32.5	17	42.5	30	37.50		
Total	40	100	40	100	80	100.00		

**** Significant at 1 %**

(Statistically significant)

Graph 6: Dentin-pulpal integrity

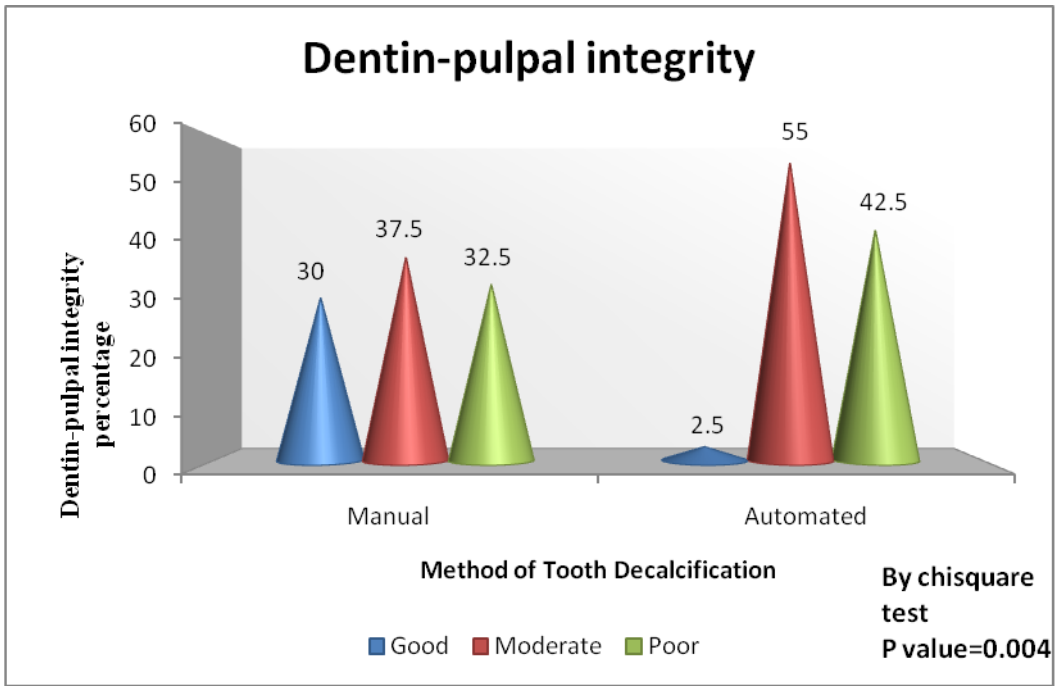


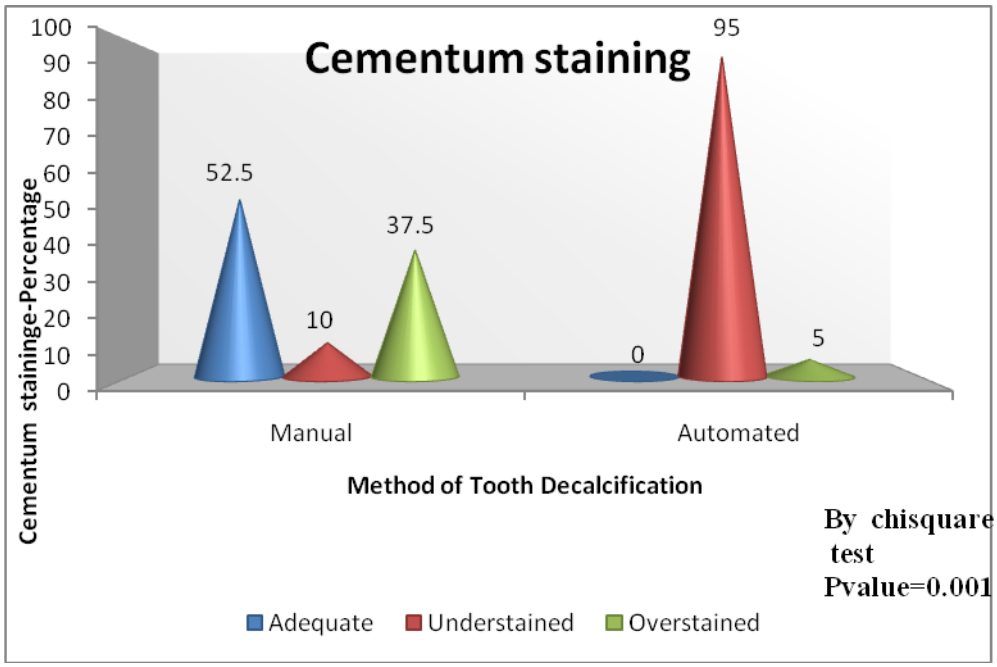
Table 7: Cementum staining

Cementum staining	Method of Tooth Decalcification				Total		Chi square	p
	Manual		Automated		N	%		
	n	%	n	%				
Adequate	21	52.5			21	26.25	58.46	0.001**
Understained	4	10	38	95	42	52.50		
Overstained	15	37.5	2	5	17	21.25		
Total	40	100	40	100	80	100.00		

**** Significant at 1 %**

(Statistically highly significant)

Graph 7: Cementum staining



RESULTS

In the methods of decalcification, while comparing the manual and automated methods, the difference in the duration of decalcification in days was statistically significant at 1% (pvalue=0.001) and in the automated method, decalcification duration is shorter (4.1days) than in manual method (7.4 days).

The ease of sectioning, when assessed in both manual and automated method of decalcification by using chi square test was statistically insignificant, indicating that there is no difference in between the two methods in ease of sectioning.

The dentinal structure were observed under microscope as clear or not clear, after decalcification with manual and automated methods, and it was found that there is no difference in both the methods (p value = 0.073) in the observation of the dentinal structures.

The dentinal staining was graded as adequate, under stained and over stained for observation and found that dentinal staining using manual method was more satisfactory than the automated method, where under staining (p value = 0.001) was observed more.

Similarly, in pulp-cellular details, the haematoxylin staining and eosin staining were graded as adequate, under stained, and over stained and observed. Manual method was better in both Haematoxylin (p value = 0.001) staining and Eosin staining (p value=0.001) and the automated method showed under staining in most of the cases.

The Dentin-pulp integrity was graded as Good, Moderate, Poor and observed, where manual method was found to be better (p value= 0.004).

The cementum staining was also better using manual method and the automated method presented with more under stained specimen(p Value=0.001).

Fig 4 : Chemical end point determination

Fig 5 : Ease of sectioning

EVALUATION OF DENTINAL STRUCTURES

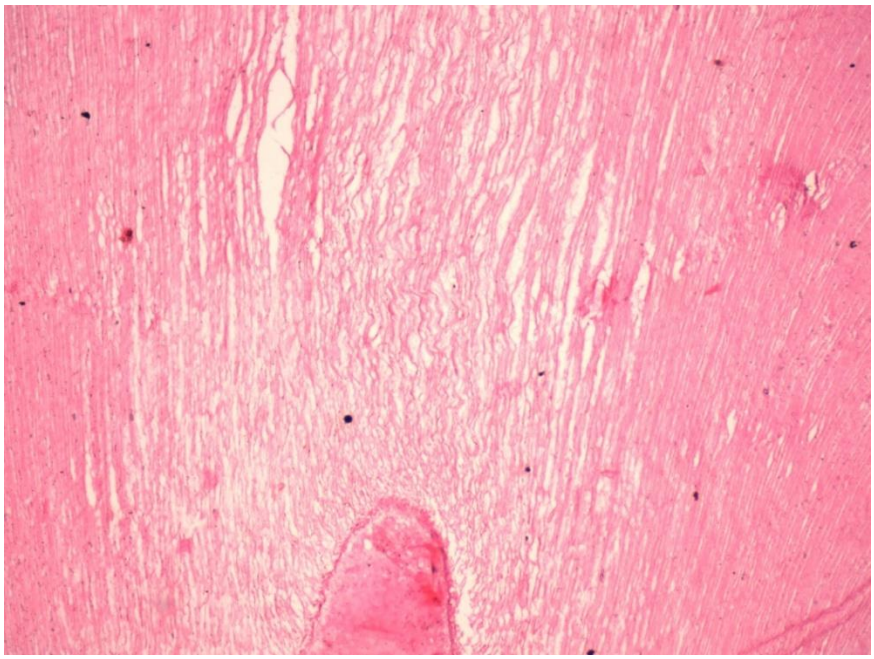


Fig 6: Dentinal structure graded as clear, H&E stain, 20x

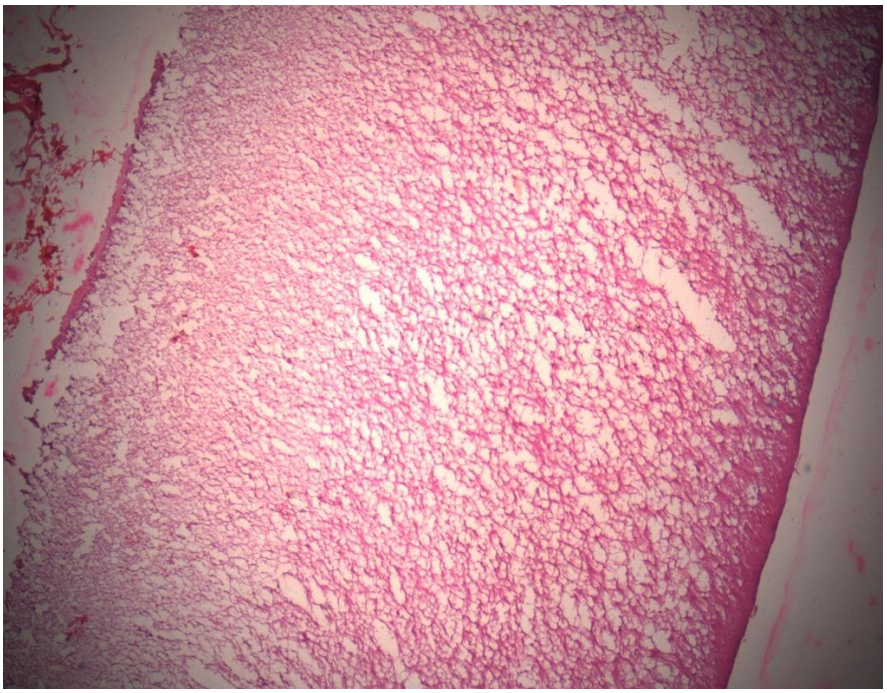


Fig 7: Dentinal structures graded as unclear, H&E stain, 20x

EVALUATION OF DENTIN STAINING

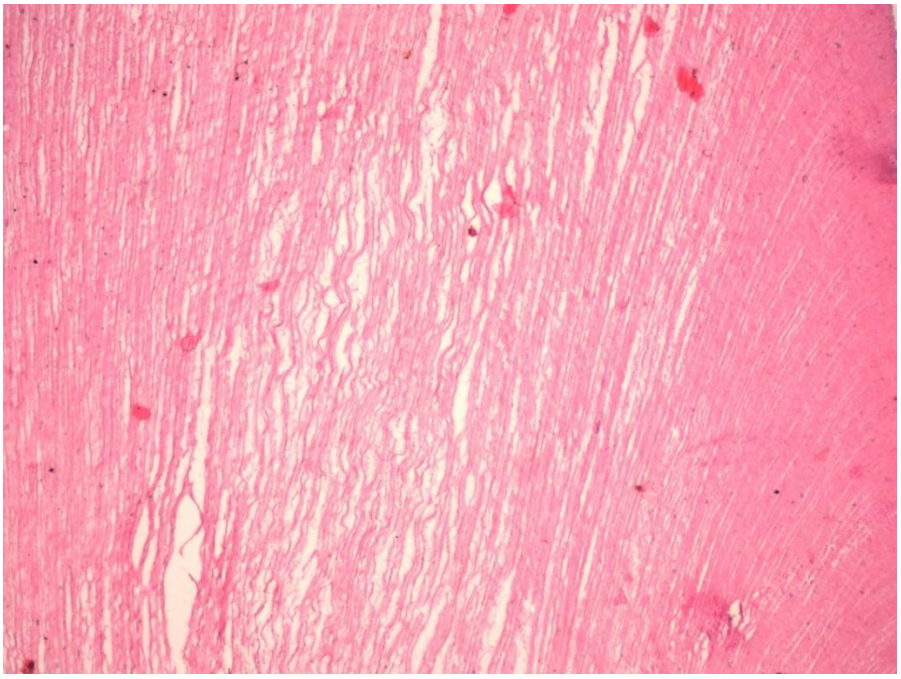


Fig 8: Dentinal staining graded as adequate, H&E stain, 40x

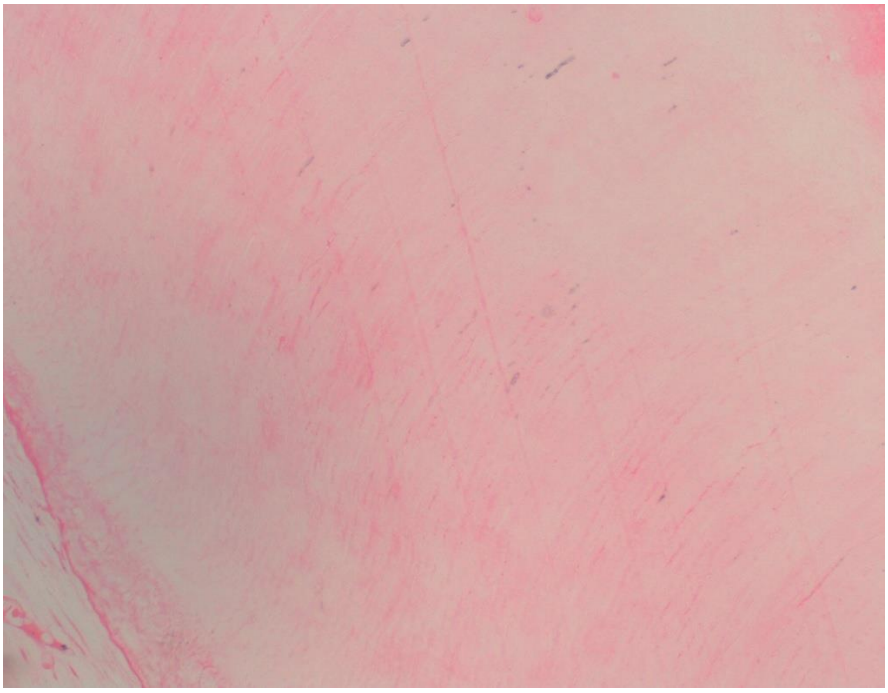


Fig 9: Dentinal staining graded as under stained, H&E stain, 40x

EVALUATION OF DENTIN STAINIING

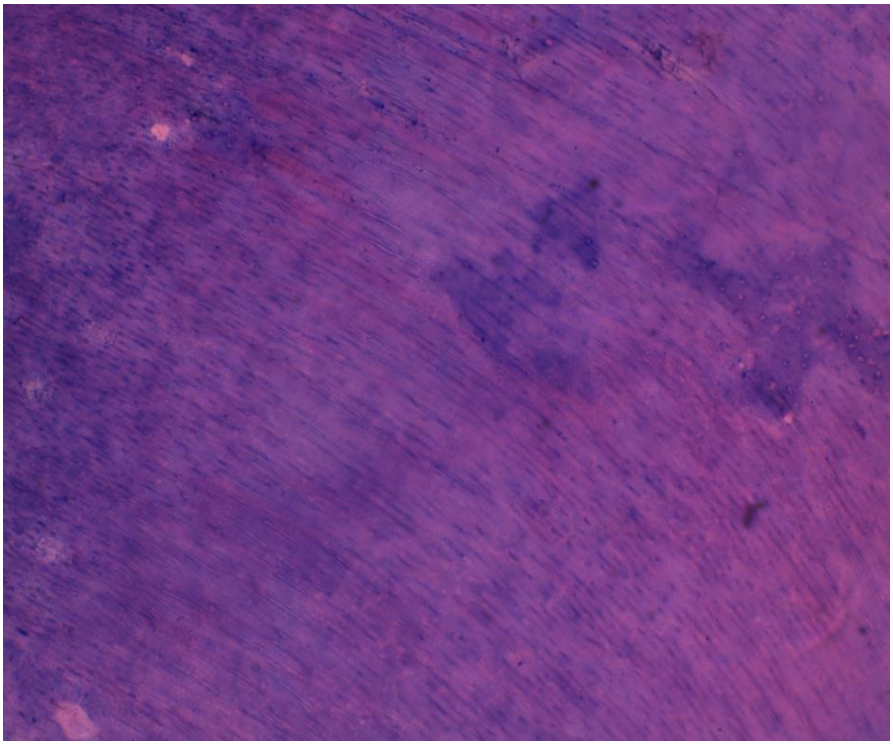


Fig 10: Dentinal staining graded as overstained,H&E stain, 40X

PULP-CELLULAR DETAILS-HAEMATOXYLIN STAINING

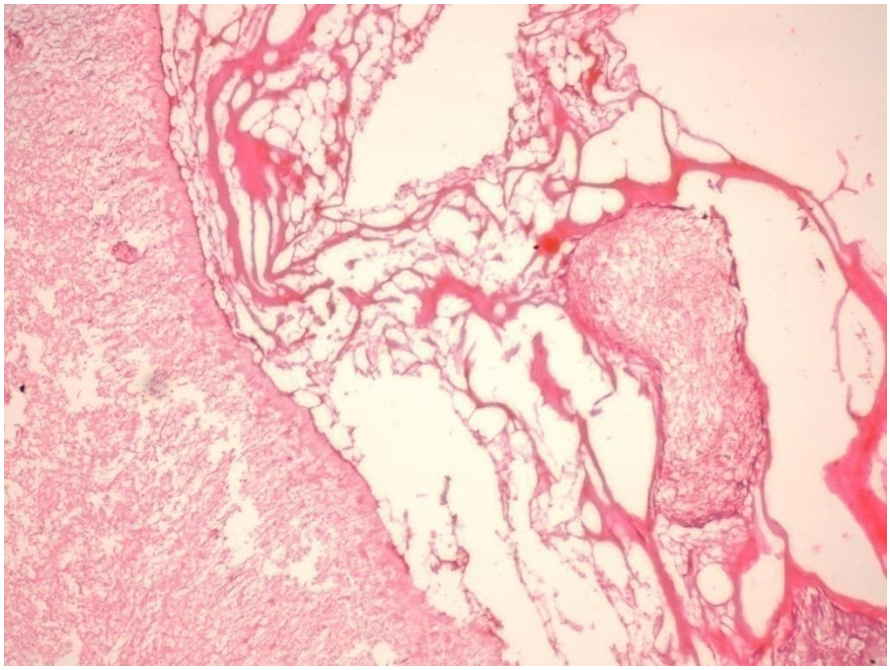


Fig 11: Pulp-cellular details graded as under stained for Hematoxylin stain, H&E stain,40x

PULP-CELLULAR DETAILS-EOSIN STAINING

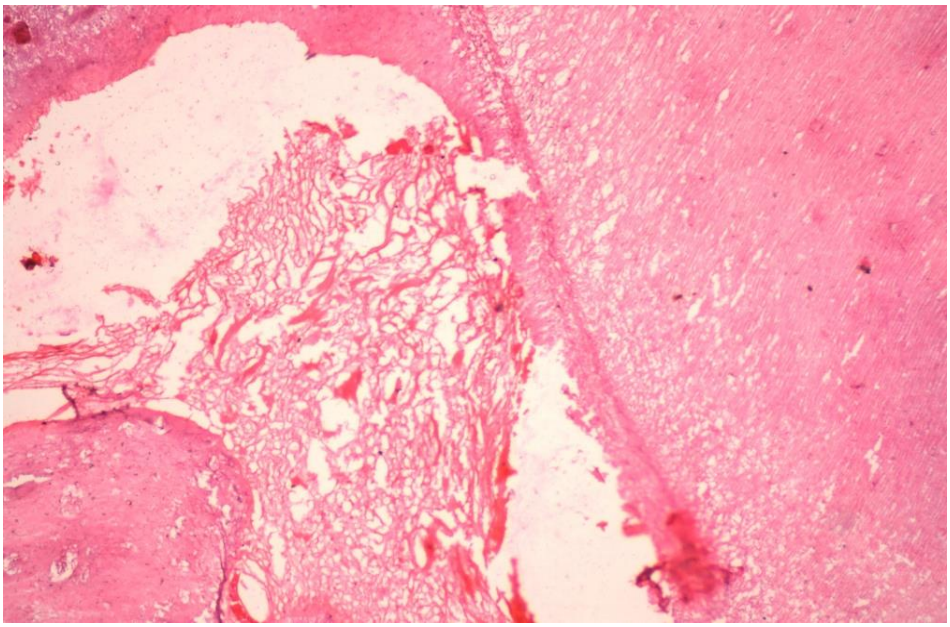


Fig 12: Pulp-cellular details graded as adequate for Eosin stain,H& E stain, 40x

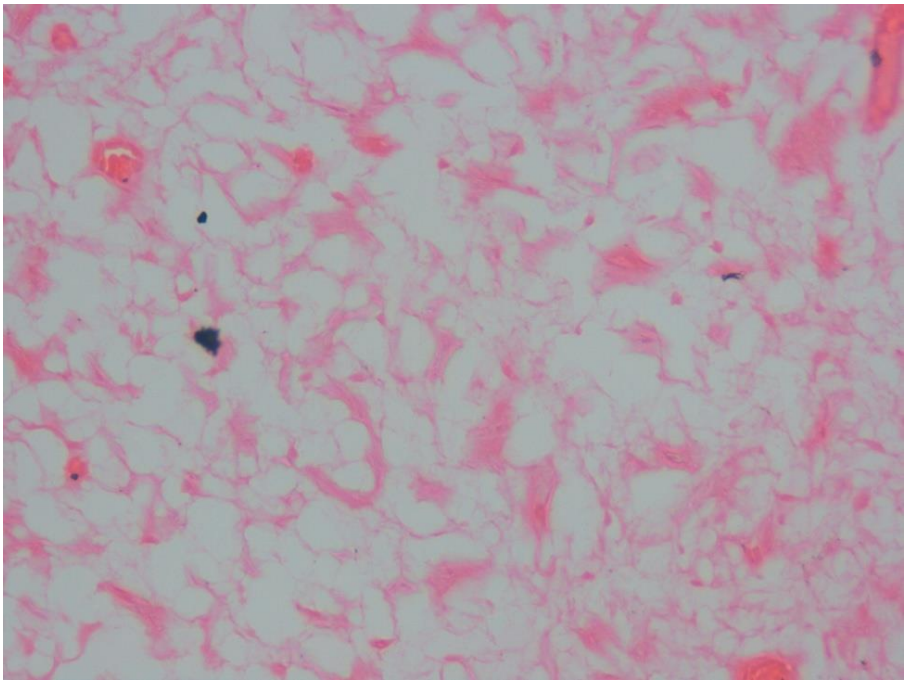


Fig 13: Pulp-cellular details graded as understained for Eosin stain, H& E stain, 40x

PULP-CELLULAR DETAILS-EOSIN STAINING

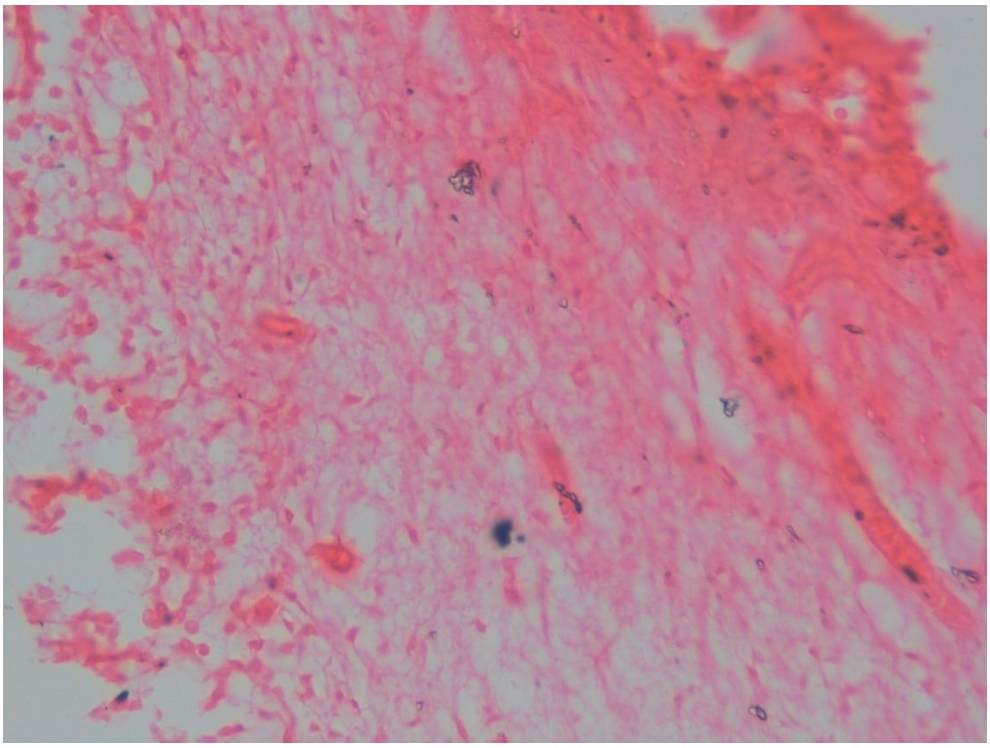


Fig 14: Pulp-cellular details graded as over stained for Eosin stain, H& E stain, 40x

EVALUATION OF DENTIN-PULP INTEGRITY

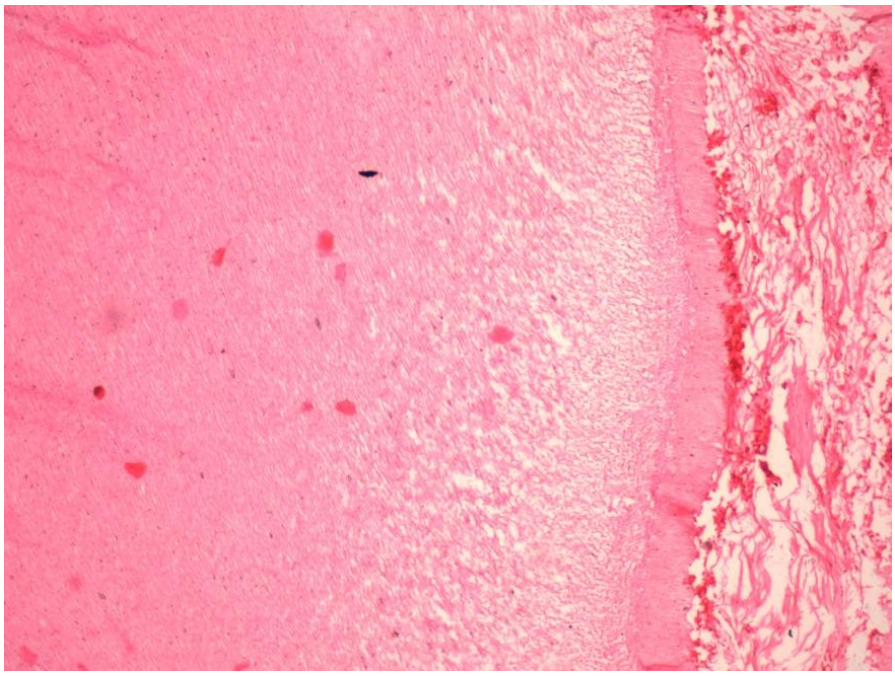


Fig 15: Dentin- Pulp integrity graded as good, H&E stain, 40x

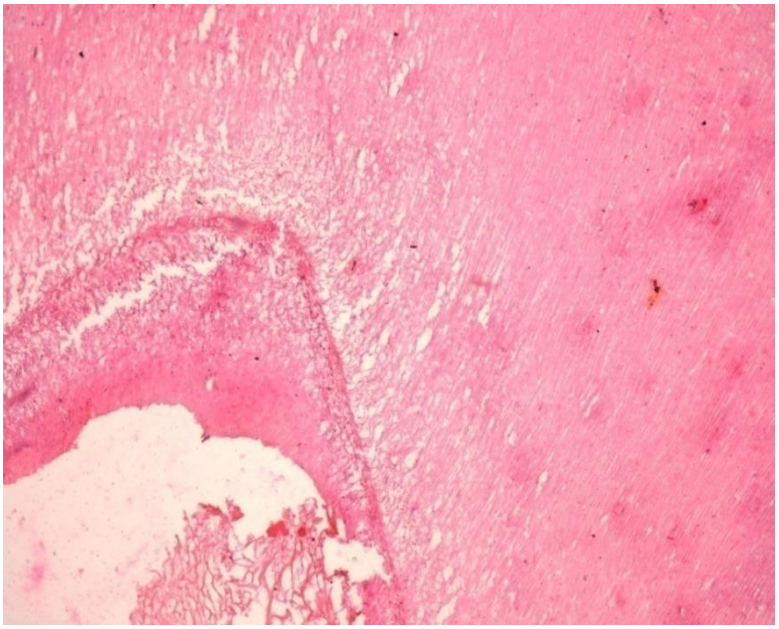


Fig 16: Dentin- Pulp integrity graded as moderate, H&E stain, 20x

EVALUATION OF DENTIN-PULP INTEGRITY

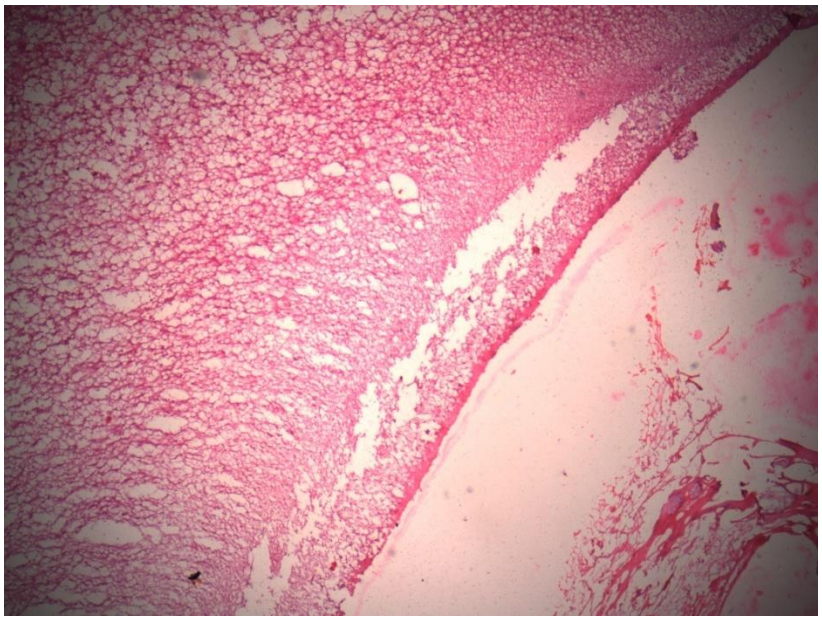


Fig 17: Dentin- Pulp integrity graded as poor, H&E stain, 20x

EVALUATION OF CEMENTUM STAINING

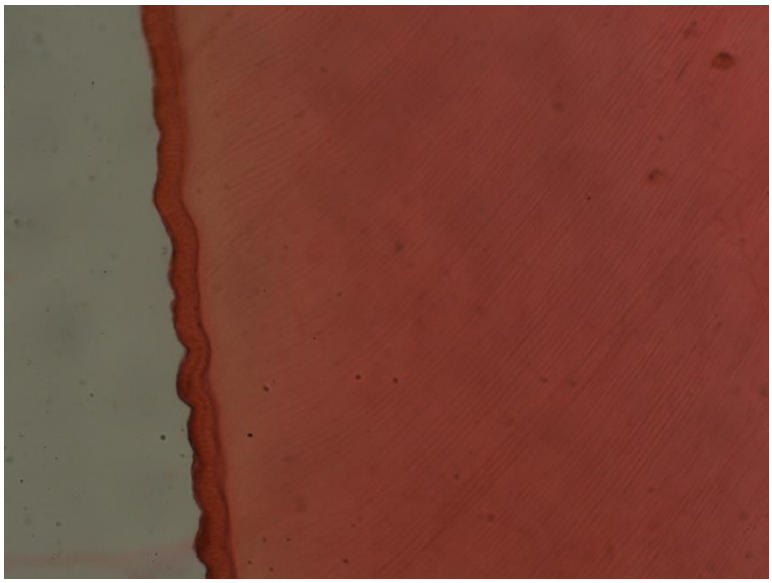


Fig 18.cementum staining graded as under stained ,H&E, 40x

DISCUSSION

DISCUSSION

Decalcification is the most important step in the preparation of oral calcified tissues for microscopic examination. It is important from two stand points. First, sections of teeth are difficult to obtain without the removal of calcium, and second, the effect of the various chemical decalcifiers upon the tissue components differs.

Preparation procedure of hard teeth specimens starts from its fixation. Choice of a fixing reagent is dependent upon the tissue itself and the purpose for which it is to be preserved. Fixation with 10% formalin seems to preserve the pulp tissue and maintain favourable conditions for examination and microscopic analysis of its cell components. 10% neutral buffered formalin is more commonly used because formalin is powerful as well as rapid in its penetration, more readily available and may be stored for longer periods. Thus, here we used 10% neutral buffered formalin to fix the tissues in our study.[1,16]

Histological sections are affected by many other variables like processing, cutting technique, staining time, etc. All the above variables were kept constant in the present study by the use of standardized techniques and recommended parameters.

DURATION OF DECALCIFICATION

Decalcification is actually the destruction of an area of tooth by constant bathing in acid. The time required for decalcification of histologic specimens must be considered a technical problem of great importance.[10] The method of end point determination is a tool for quantitative as well as qualitative evaluation of decalcification methods and should be precisely done to determine the time required for decalcification.[11] So, we depended both on radiographic as well as chemical

methods for determining the end point of decalcification. Finally, number of days taken for decalcification is calculated after excluding the holidays (if any) in between the decalcification process, and in those holidays, the decalcified specimens were transferred to the water to avoid overdecalcification. Thus, in our study, decalcification is achieved within average of 7.4 days in manual method, which is the longest and the same is 4.1 days in automated method, which is the fastest. With the automated method in our study, there is a constant bathing of tooth specimen by acids on all sides by vibration, which increases the rate of penetration, thereby decreasing the time taken for decalcification. According to Murayama and his colleagues(1937), with increasing temperature, there will be a gradual decrease in the time necessary for decalcification.[1]

EVALUATION OF EASE OF SECTIONING

The ease of sectioning obtained by both decalcification methods is graded as easy, difficult and very difficult and its percentage are 45%,27.5%,27.5% respectively in manual method. The grades for ease of sectioning obtained by automated method are 52.5% (easy),30% (difficult),and 17.5% (very difficult). The results of our study showed that there is no difference in ease of sectioning between manual and automated methods. This could be because, we have used the same decalcifying agent (8% formal nitric acid) in both the methods and a previous study finds difference in ease of sectioning with the usage of different decalcifying agents.[4] Also the processing of decalcified specimens after both the methods of decalcification was similar, thereby leading to this insignificant statistical difference. Thus we infer that the method of decalcification persay, does not cause any effect on ease of sectioning.

DENTINAL STRUCTURE INTERPRETATION

Mineralised dentin is separated from the odontoblast layer by the predentin, a 10 to 40 micrometre thick layer of unmineralised matrix.[54] Based on the fraying in the dentinal tubules and presence of vapor bubbles, destruction of odontoblast layer, the harmful effects of the decalcifying solutions on dentin are assessed. Acid attacks may also causes zone of decomposed dentin. The Dentinal structure obtained by manual methods was graded as clear and not clear and its percentage are 57.5%,and 42.5% respectively where as in automated methods the percentage obtained are 37.5% and 62.5% respectively and on comparison, it shows manual method to be better.

The destruction of dentin by decalcification and then proteolysis have occured in numerous focal areas leading to necrotic mass of dentin, dentin destructions showing vapour bubbles and dentin fraying. Previous studies suggest that strong acid decalcification opened up the dentinal tubules quickly and serve as a pathway to the pulp tissue, thus destroying or separating the pulp from dentin. That is why, rapid decalcification in automated method, cause more fraying in the dentinal tubules along with more destruction of the odontoblasts architecture.[4]

DENTINAL STAINING

The dentinal staining obtained by manual methods is graded as adequate, under stained and over stained and the percentage obtained respectively are 47.5%,2.5%,and 50% whereas in automated methods are 32.5% and 62.5% and 5% respectively. When compared to both results, it shows that manual method to be with better efficacy. Under staining is more prominent in the automated method, which may be attributed to the rapid action of the acid impairing the hematoxylin intake. The most pronounced effect of acid decalcification is the impairment of staining

properties. According to Stevans et al(1990) and callis and sterchi (1998), strong acids such as nitric acid can decalcify rapidly, but cause serious deterioration of stainability, which is dependent on the solution acidity and the time it will take to decalcify. Thus, we suggest, prolonged water wash after automated method of decalcification may improve the H&E staining.

PULP CELLULAR DETAILS – HAEMATOXYLIN STAIN

Pulp cellular details are evaluated and the effect of decalcification methods on pulpal staining is assessed by intensity of hematoxylin staining of the nuclei and intensity of eosin staining of the cytoplasm. H&E staining is assessed and graded as adequate or over stained or under stained. Haematoxylin stain obtained by manual methods, graded as adequate, under stained, and over stained are with the percentage of 50%,7.5%,and 42.5% respectively whereas in automated methods, it was 5% and 95% respectively with no over staining (0%). When compared to both the methods, the manual method is found to show better efficacy.

PULP CELLULAR DETAILS – EOSIN STAIN

The Eosin stain obtained by manual methods are graded as adequate, under stained, and over stained and the percentage obtained are 50%, 10 % and 40% respectively whereas in automated methods, the percentage obtained are 32.5%, 62.5% and 5% respectively. Here also, when compared to both the methods, manual method shows better efficacy. Histochemical studies on demineralised material have been limited because of the interference of acid with H&E staining.[47-50] The effects on H &E staining after automated method of decalcification can be reduced by improved or prolonged methods of post decalcification acid removal, and adjustment of the staining procedures.[42]

DENTIN PULP INTEGRITY

Dental pulp evaluation is the most sensible part of research protocols followed in assessment of decalcified sections[4], because the pulp contains the soft tissue components and is the most affected tissue during decalcification.[53]According to Sanjai et al (2012) histological examination of hard dental tissue and pulp is imperative for diagnosis of developmental disorders, pulp pathologies, forensic odontology and research.[17]In a perfectly decalcified section, the relation between pulp tissue and dentin should be maintained allowing for simultaneous observation of both structures.[26]In our present study, the dental pulp was examined for the presence of all the four zones of the pulp and the amount of separation of pulp from the surrounding dentin.[4]The Dentin-pulp integrity obtained by manual methods are graded as good, moderate, poor and percentage obtained are 30%, 37.5%and 32.5% respectively whereas in automated methods the results obtained are 2.5%, 55% and 42.5% respectively. When compared to both the methods, manual methods maintains the dentin-pulp integrity better and in the automated method, the percentage of decalcified specimens with good dentin-pulp integrity is very less (2.5%).

Pulp separation from dentinal border and preservation of cellular details is dependent on fixation and the choice of decalcifying agents. Frequently, obtaining a good histological result for the calcified tissues is not possible without some damage to the soft tissues. On the other hand, adequate preservation of the soft tissues leaves the specimen incompletely decalcified. Soft tissues put forth little resistance to the histochemical techniques and the lesions affecting hard tissues need intricate, technique sensitive methodology for interpretation and diagnosis.[2]According to

Fernandes et al, (2007) it is difficult to fulfill the requirements for simultaneous analysis of mineralised and non-mineralised tissues.

Thus, from our study results, with the usage of strong acids like nitric acid, manual method is better in providing better dentin – pulp integrity than the automated method. Usage of chelating agents like EDTA in the automated machine can improve the soft and hard tissue integrity.

CEMENTUM STAINING

On assessment of cemental staining, the staining is graded as adequate, under stained and over stained and the results obtained with manual method are 52.5%, 10% and 37.5%respectively whereas in automated methods, the results obtained are 95% and 5% (under stained and over stained) with no adequately stained cementum (0%) in decalcified specimen. The deterioration in cemental staining destruction, more commonly in automated method could be explained on the basis of lytic effects of the acids.

SUMMARY &CONCLUSION

SUMMARY & CONCLUSIONS

Decalcification of hard tissue specimen is a very technique sensitive method and plays an important role in oral pathology, as decalcification of bone or teeth is done on a regular day to day basis for histopathology reporting. In our study,8% formal nitric acid showed the most efficient result as it balances both tissue integrity and time factor suggesting that it can be used as a stable decalcifying agent for routine histopathological diagnosis. This study is a step forward in establishing the decalcification dynamics and identifying the method that combines the highest quality of stained sections with the shortest time.

Thus, from our study results,

1) Duration of days taken for decalcification proved to be found better in automated method of decalcification.
2) Ease of sectioning shows no difference between manual and automated methods of decalcification.
3) Dentinal structure is more clearly seen in manual method and manual method preserves the structural details in a better way than the automated method
4) Dentinal staining is more satisfactory in manual method of decalcification. Under staining is the most commonly seen demerit after automated method of decalcification.
5) Haematoxylin staining in pulpal structures are evaluated to be better in manual method of decalcification
6) Eosin staining in pulpal zones are better in manual method when comparing to the automated method.

7) Dentin - pulpal integrity is preserved better in manual method of decalcification than in automated method.
8) Manual method of decalcification proves to be better in cementum staining.

Thus, we recommend automated method of decalcification in the histopathological practice for time bound diagnosis and prolonged water wash after automated method of decalcification may improve the H&E staining of various dental structures. Further studies with different decalcifying agents in larger scale is recommended.

REFERENCES

REFERENCES

1. Prasad P, Donoghue M. A comparative study of various decalcification techniques. Indian Journal of Dental Research 2013;24(3):302-308.
2. Srinivasyaiah A, Nitin P. and Hegde U.Comparison of microwave versus conventional decalcification of teeth using three different decalcifying solutions. Journal of Laboratory Physicians 2016; 8(2):106-111.
3. Afreen N. Decalcification: a simpler and better alternative. Journal of dentistry and oral biosciences 2011;2(2):10-13.
4. Kapila S. Driving the Mineral out Faster: Simple Modifications of the Decalcification Technique. Journal of clinical and diagnostic research 2015;9(9):93-98.
5. Sanjai K, Patil A, Jayaram S, Kumarswamy J, Papaiah L, Krishnan L. Evaluation and comparison of decalcification agents on the human teeth. Journal of Oral and Maxillofacial Pathology 2012;16(2):222-227
6. Choube A, Astekar M, Choube A, Sapra G, Agarwal A, Rana A. Comparison of decalcifying agents and techniques for human dental tissues. Biotechnic & Histochemistry 2018;93(2):99-108.
7. Vongsavan N, Matthews B, Harrison G. Decalcification of teeth in a microwave oven. The Histochemical Journal 1990;22(6-7):377-380.
8. Verdenius H, Alma L. A quantitative study of decalcification methods in histology. Journal of Clinical Pathology 1958;11(3):229-236.
9. Naik R, Pai M, Rai S, Baliga P, Mathai A. Microwave histoprocessing versus conventional histoprocessing. Indian Journal of Pathology and Microbiology 2008;51(1):12-16.

10. Kok L, Visser P, Boon M. Histoprocessing with the microwave oven: an update. The Histochemical Journal 1988;20(6-7):323-328.
11. Simmelink, J. and Abrigo, S.. Crystal Morphology and Decalcification Patterns Compared in Rat and Human Enamel and Synthetic Hydroxyapatite. Advances in Dental Research 1989:(2):241-248
12. Leong A. Microwaves and Turnaround Times in Histoprocessing. American Journal of Clinical Pathology 2004;121(4):460-462.
13. Leong A, Sormunen R. Microwave Procedures for Electron Microscopy and Resin-Embedded Sections Micron 1998;29(5):397-409.
14. Fernandes, M., Gaio, E., Rosing, C., Oppermann, R. and Rado, P. Microscopic qualitative evaluation of fixation time and decalcification media in rat maxillary periodontium. Brazilian Oral Research 2007;21(2):134-139.
15. Zappa J,Bielecka A,Adwent M,Cieslik K,Sabat D,Comparison of different decalcification methods to hard teeth tissues morphological analysis. Dent Med Probl 2005:42(1):21-26.
16. Gupta S. Qualitative Histological Evaluation of Hard and Soft Tissue Components of Human Permanent Teeth Using Various Decalcifying Agents - A Comparative Study. Journal of clinical and diagnostic research 2014: 8(9): 69-72.
17. Warshawsky H, moore G. A technique for the fixation and decalcification of rat incisors for electron microscopy. Journal of Histochemistry & Cytochemistry 1967;15(9):542-549.
18. Coleman E, Desalva S. Rapid Decalcification for Histochemistry. Journal of Dental Research 1966;45(4):1236-1237.

19. Mardfin D, James V. Effect of Nitric Acid and Chelation Demineralization on Various Stains of Histochemical Nature. Journal of Dental Research 1957;36(5):759-764.
20. Lillie R, Laskey A, Greco J, Burtner H, Jones P. Decalcification of bone in relation to staining and phosphatase technics. American Journal of Clinical Pathology 1951;21(8):711-722.
21. Schajowicz F, Cabrini R. The effect of acids (decalcifying solutions) and enzymes on the histochemical behavior of bone and cartilage. Journal of Histochemistry & Cytochemistry 1955;3(2):122-129.
22. Thorell B, Wilton À. The nucleotide metabolism of the dentine cells under normal conditions and in avitaminosis c. Acta Pathologica Microbiologica Scandinavica 2009;22(6):593-602.
23. Wislocki G, Sognnaes R. Histochemical reactions of normal teeth. American Journal of Anatomy 1950;87(2):239-275.
24. Birkedal-Hansen K. Kinetics of acid demineralization in histologic technique. Journal of Histochemistry & Cytochemistry 1974;22(6):434-441.
25. Cook S, Ezra-cohn H. A comparison of methods for decalcifying bone. Journal of Histochemistry & Cytochemistry 1962;10(5):560-563.
26. Bourque W, Gross M, Hall B. A histological processing technique that preserves the integrity of calcified tissues (bone, enamel), yolky amphibian embryos, and growth factor antigens in skeletal tissue. Journal of Histochemistry & Cytochemistry 1993;41(9):1429-1434.
27. Laboux O, Dion N, Arana-Chavez V, Ste-Marie L, Nanci A. Microwave Irradiation of ethanol-fixed bone improves preservation, reduces processing time,

and allows both light and electron Microscopy on the same sample. Journal of Histochemistry & Cytochemistry 2004;52(10):1267-1275.

28. Goland P, Scheiman-Tagger E, Engel M. Enamel preservation during decalcification following fixation by some reactive halogen compounds. Journal of Dental Research 1965;44(2):342-349.
29. Boon M, Kok L, Ouwerkerk-noordam E. Microwave-stimulated diffusion for fast processing of tissue: reduced dehydrating, clearing, and impregnating times. Histopathology 1986;10(3):303-309.
30. Smith C. Effect of glutaraldehyde and decalcifying agents on acid phosphomonoester hydrolase activity in the enamel organ of the rat incisor: a biochemical study comparing enamel organ with liver. Journal of Histochemistry & Cytochemistry 1980;28(7):689-699.
31. Kok L, Boon M. Physics of microwave technology in histochemistry. The Histochemical Journal 1990;22(6-7):381-388.
32. Pitol D, Caetano F, Lunardi L. Microwave-induced fast decalcification of rat bone for electron microscopic analysis: an ultrastructural and cytochemical study. Brazilian Dental Journal 2007;18(2):153-157.
33. Reith E, Boyde A. The pyroantimonate reaction and transcellular transport of calcium in rat molar enamel organs. Histochemistry1985;83(6):539-543.
34. Massa L, Bradaschia-Correa V, Arana-Chavez V. Immunocytochemical study of amelogenin deposition during the Early odontogenesis of molars in alendronate-treated newborn rats. Journal of Histochemistry & Cytochemistry 2006;54(6):713-725
35. Stan PH. Calcified tissue. In: Boon ME, Kok LP (Editors)MicrowaveCookBook of Pathology. Coulomb Press Leynden: Leinden 1988: p.264-266.

36. Wagenaar F, Kok GL, Broekhuijsen-Davies JM, Pol JMA. Rapid cold fixation of tissue samples by microwave irradiation goes it uses in electron microscopy. Histochem J 1993;25:719-725.
37. Callis GM, Bancroft JD. Theory and Practice of Histological Techniques. 6th ed. Edinburgh: Churchill Livingstone; 2008; p. 338-360.
38. Babu T, Malathi N, Magesh K. A comparative study on microwave and routine tissue processing. Indian Journal of Dental Research 2011;22(1):50-55
39. Muller S, Pleul J, Gotz M, Jahnig J, Schadlich H. A method to determine the end point of decalcification of hard tissue and bone. Stain Technol 1990;65: 77–83.
40. Mattuella LG, Bento LW, Vier – Pellisser FV, Araiyo FB, Fossati AC. Comparative analysis of two fixing and two decalcifying solutions for processing of human primary teeth with inactive carious lesion. Rev odontocienc 2007;22:99-105.
41. Waerhaug J. Decalcification of Bone and Teeth Under Vacuum- A Rapid Method for producing hard tissue preparations. Journal of Dental Research 1949;28(5):525-526.
42. Morse A. Formic acid-sodium citrate decalcification and butyl alcohol dehydration of teeth and bones for sectioning in paraffin. Journal of Dental Research 1945;24(34):143-153.
43. Selvig K. Ultrastructural changes in human dentine exposed to a weak acid. Archives of Oral Biology 1968;13(7):719-734.
44. Crespi R, Grossi S. A Method for histological examination of undecalcified teeth. Biotechnic & Histochemistry 1992;67(4):202-206.

45. Uma K, Chandavarkar V, Sangeetha R. Comparison of routine decalcification methods with microwave decalcification of bone and teeth. Journal of Oral and Maxillofacial Pathology 2013;17(3):386.
46. Mawhinney W, Richardson E, Malcolm A. Control of rapid nitric acid decalcification. Journal of Clinical Pathology 1984;37(12):1409-1413.
47. Morales A, Nassiri M, Kanhoush R, Vincek V, Nadji M. Experience with an automated microwave-assisted rapid tissue processing method. American Journal of Clinical Pathology 2004;121(4):528-536.
48. Kayser K, Stute H, Lubcke J, Wazinski U. Rapid microwave fixation?a comparative morphometric study. The Histochemical Journal 1988;20(6-7):347-352.
49. Donath K, Breuner G. A method for the study of undecalcified bones and teeth with attached soft tissues. The Sage-Schliff (sawing and grinding) technique. Journal of Oral Pathology and Medicine.1982;11(4):318-326.
50. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 5th ed. Missouri: Harcourt Publishers;2002.
51. Culling's CF, Allison RT, Histological Technique Processing. 4th ed. London : Butterworths and Co-publishers; 1985. p. 51-77 .
52. Carletons HM,and Drury RAB,Histological techniques,3rd ed.,Oxford university press,New York 1957.
53. Cook DJ. In Cellular pathology: Introduction to techniques and applications. 2nd ed. Oxfordshire: Scion; 2006. p. 31–36.

ANNEXURE

INSTITUTIONAL ETHICS COMMITTEE
VIVEKANANDHA DENTAL COLLEGE FOR WOMEN

SPONSORED BY : ANGAMMAL EDUCATIONAL TRUST

Ethics Committee Registration No. ECR/784/Inv/TN/2015 issued under Rule 122 DD of the Drugs & Cometics Rule 1945.

Dr. J. Baby John	**Chair Person**	**Dr. (Capt.) S. Gokulanathan**	**Member Secretary**
Mr. K. Jayaraman	Social Scientist	Mr. A. Thirumoorthy	Legal Consultant
Dr. R. Jagan Mohan	Clinician	Dr. N. Meenakshiammal	Medical Scientist
Dr. B.T. Suresh	Scientific Member	Dr. R. Natarajan	Scientific Member
Dr. Sachu Philip	Scientific Member	Mr. Kamaraj	Lay Person

No: VDCW/IEC/ 54/2017 Date: 30.12.2017

TO WHOMSOEVER IT MAY CONCERN

Principal Investigator: Dr. K.Gayathri

Title: Comparison between Manual and Automated method of decalcification- an Invitro study.

Institutional ethics committee thank you for your submission for approval of above proposal .It has been taken for discussion in the meeting held on 22.12.17. The committee approves the project and it has no objection on the study being carried out in Vivekanandha Dental College for Women.

You are requested to submit the final report on completion of project. Any case of adverse reaction should be informed to the institutional ethics committee and action will be taken thereafter.

CHAIRMAN
INSTITUTIONAL ETHICS COMMITTEE
VIVEKANANDHA
DENTAL COLLEGE FOR WOMEN
Elayampalayam-637 205.
Tiruchengode (Tk) Namakkal (Dt),
Tamilnadu.

SECRETARY
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